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HOST-MICROBE-DIET INTERPLAY: DIETARY MODULATION OF THE GUT MICROBIOTA IN RELATION TO HEALTH

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by

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Vertebrates are associated with trillions of bacteria, with the densest populations residing in the large intestine. The symbiosis between vertebrates and their gut microbiota has resulted in important implications of the gut microbiome on host health. Diet is an important factor that shapes gut microbiota composition, and because of the interplay between host-microbiome-diet, dietary strategies that modulate gut microbiome structure are deemed a relevant tool to improve host health. However, gaps in knowledge exist with respect to these interactions, and it is essential to obtain a mechanistic understanding of how these relations take place to develop successful therapeutic strategies that target the gut microbiome.

In order to address these gaps, human trials were performed to assess the impact of primary components of the human diet, resistant starches and whole grains, on the gut microbiota. Overall, the impact of diet was temporal and varied across subjects. Resistant starches substantially modulated the gut bacterial community of the subject population, especially increasing the abundance of *Bifidobacterium adolescentis*. *Ruminococcus bromii*, *Eubacterium rectale*, and *Parabacteroides distasonis* were also significantly enriched. Dietary incorporation of whole grains increased the proportions of *Eubacterium rectale* and acetogens such as *Blautia wexlerae*. Of note, whole grains significantly improved inflammation and glycemic parameters. The shifts in *Eubacterium rectale* correlated with glycemic improvements. Moreover, distinct abun-

dances of *Dialister* were determined among subjects that differed in terms of their inflammatory improvement.

To gain mechanistic insight on the host-microbe-diet interplay, animal experiments were conducted to evaluate the effects of grain sorghum lipids and plant sterol esters in the context of dyslipidemia. Significant and consistent alterations in gut microbiota composition were detected in both experiments, especially involving shifts in Coriobacteriaceae and Erysipelotrichaceae abundance, which displayed remarkable correlations to host cholesterol markers. Mathematical modeling of these associations revealed them to be inhibitory interactions, suggesting that changes in host metabolism affected gut microbiome structure through an antimicrobial effect of cholesterol, which was confirmed *in vitro* against selected gut microbes.

In conclusion, the studies presented in this dissertation allowed new insights on the impact of diet on the gut microbiota and its consequences for health.

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Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Host-microbe symbiosis in the gastrointestinal tract

The microbial community associated to the gastrointestinal tract[1] (GIT) is characterized by a high population density, ample diversity and great complexity in terms of interactions and dynamics. The gut microbiota (GM) is composed of a broad repertoire of viruses, bacteria and to a lesser degree archea, fungi and yeasts[2], with the bacterial cells in the GIT outnumbering our own cells by at least an order of magnitude.

The relationship between vertebrates and their gut microbiota can be referred to as a symbiosis. A symbiotic relationship is defined as a close and long-term association among species (de Bary, 1879), and can potentially develop into mutually beneficial interactions in which the members enhance their partner's fitness. The mutualisitc nature of the vertebrate/microbiome symbiosis is exemplified by the important role of bacteria in their host's biology. The gut microbiota contributes to nutrient provision and energy storage, prevention of pathogen colonization (colonization resistance), and the development of host physiology and immune functions (Gordon and Pesti, 1971, Reddy et al., 1972, Savage, 1977).

The relevance of the GM in vertebrate biology is evidenced by the fact that several species would not survive or would have impaired growth without a functional microbiome. For example, a herbivorous lifestyle based on fibrous plants would not be possible as vertebrates do not possess the enzymes to digest cellulose and hemicellulose (Flint et al., 2008). Accordingly, some species, such as koalas and hamsters, cannot even be reared germ-free (GF) (Stevens and Hume, 1998, Pollard, 1976).

[1]For the purposes of this thesis, the main focus will reside on the small and large intestine

[2]For the purpose of this thesis, the discussions will be centered upon the bacterial component of the gut microbiota.

Even in vertebrates that do not have a strictly obligate relationship with the GM, the important contributions of the microbiome in nutrient provision and resistance to infections are still essential for the species' success. The GM provides the host with nutrients in the form of short-chain fatty acids (SCFAs) resulting from the fermentation of non-digested carbohydrates that are absorbed by the host. In the case of herbivores (e.g.: ruminants), the bacterial contribution to the animal's energy intake is approximately 70%, and in omnivores such as humans is around 10% (Flint, 2011). Acetate, propionate and butryrate are the principal SCFAs in the GIT, and have different metabolic fates. Acetate and propionate are mainly taken up by the liver where they undergo gluconeogenesis and lipogenesis (Turnbaugh et al., 2006), while butyrate is the principal energy source of colonic epithelial cells (Louis et al., 2007).

1.2 Maintenance and control of microbial populations in the gastrointestinal tract

Given the importance of the GM for vertebrate biology, the host must maintain and control these bacterial populations to maximize the benefits of this symbiosis. The restricted enzymatic capability of humans to degrade complex carbohydrates allows for non-digestible carbohydrates (NDC) to escape digestion and reach the large intestine where they can be fermented by the resident bacteria (Flint et al., 2008). The GM possesses a wide repertoire of enzymes to sense, capture and hydrolyze undigested carbohydrates of diverse chemical structures (Martens et al., 2008), and it has been estimated that approximately 10% of the bacterial microbiome encodes for enzymes associated with the carbohydrate metabolism (Turnbaugh et al., 2009, Qin et al., 2010), evidencing the importance of carbohydrate metabolic processes in the GIT. In

addition, host-derived glycans represent a constant energy source for GIT bacteria (Salyers et al., 1977). This nutrient rich environment provided by the host enables the maintenance of the dense bacterial populations of the GIT.

In humans, the composition and density of the bacterial populations present in the GIT are determined by the physicochemical characteristics of the different anatomical regions (Booijink et al., 2007). Factors including peristaltic motility, pH, redox potential, host secretions (i.e.: bile acids, mucus, etc.), and available nutrients help maintain and control the bacterial populations in the GIT (Booijink et al., 2007). The low pH in the stomach severely restricts the bacterial density and diversity in the organ. About $10^{2\text{-}3}$ bacterial cells/ml of content are present in the stomach. *Helicobacter pylori* is considered the only true resident (autochthonous) bacterial species in the stomach of humans (reviewed in Walter and Ley (2011)). The duodenum harbors a low-density bacterial community of approximately $10^{3\text{-}4}$ cells/g of content, limited by the high concentration of bile acids, relatively low pH and a high transit rate characteristic of the proximal small intestine. The increase in pH in more distal regions and the reabsorption of bile acids allow a gradual increase of the microbial load throughout the small intestine to reach densities of 10^8 cells/g of content in the more distal sections. Bacterial restriction in these anatomical sites is considered important as it reduces conflict for nutrients, and maximizes substrate absorption by the host (Walter and Ley, 2011). Proteobacteria such as *Escherichia coli* and *Klebsiella* and Firmicutes and Bacteroidetes genera (*Enterococcus*, *Bacteroides*, *Ruminococcus*, *Dorea*, *Clostridium*, *Coprococcus*, *Weissella*, and some *Lactobacillus*) are autochthonous to the small intestine of humans (Walter and Ley, 2011). The ileocecal valve provides a physical and anatomical separation from the harsh environment in the small intestine and gives way to the largest bacterial reservoir in the human body, the large intestine. Availability of the non-digested nutrients, anoxic conditions, and

slow transit times in the large intestine allow for the presence of up to 10^{11} cells/g of bacteria, and make the large intestine the primary site of bacterial fermentation.

The large ensemble of microorganisms in the GIT is in great proximity to the host, and thus constitutes a constant threat of invasion. The host has therefore evolved sophisticated mechanisms to keep the trillions of bacteria associated to the GIT in check to ensure homeostasis (Hooper and Macpherson, 2010). A brief overview of the main mechanisms involved in immune homeostasis in the GIT is provided due to their importance in allowing the host-microbe mutualism to develop.

First, gut barrier function is achieved by mechanisms that prevent direct contact between the gut microbiota and the host epithelium, and constitute the first line of defense against microbial invasion. These mechanisms include the secretion of mucus, antimicrobial products, defensins and immunoglobulins (Ig), in particular IgA. Goblet cells present in the intestinal epithelium secrete mucin glycoproteins that make up the main constituent of the mucus layer. Mucins form a thick, viscous layer that preovides physical, immunological and mechanical protection from bacterial invasion (Johansson et al., 2008, Hooper and Macpherson, 2010). The importance of the mucus layer has been demonstrated in Muc2 deficient animal models, which develop spontaneous intestinal inflammation (Johansson et al., 2008). This layer is organized in two strata, an outer layer that is associated with bacteria, and an inner layer that remains basically sterile (Johansson et al., 2008). It is thought that mucins in the outer layer act as 'decoy' structures for bacterial attachment and thus contribute to the shedding of intestinal bacteria (Lindén et al., 2009). The inner mucus layer is associated with secretory antibodies (mainly IgA), that are important to keep symbiotic bacteria restricted to the intestinal lumen (Hooper and Macpherson, 2010, Macpherson et al., 2000). Dendritic cells sample for microorganisms in the GIT environment and induce B-cell differentiation into plasma cells that translocate to the intestinal

lamina propria and secrete IgA that is transcytosed across the intestinal epithelium (Fagarasan and Honjo, 2003). The inner mucus layer is also associated with antimicrobial peptides that include defensins, lectins and cathelicidins, which have a direct bactericidal effect. Antimicrobial peptides such as RegIIIγ have been proved crucial to gut homeostasis (Vaishnava et al., 2011), as RegIIIγ(-/-) mice revealed increased bacterial density associated to the epithelial surface and have an exacerbated immune response.

These mechanisms illustrate only a small fraction of the great complexity of the host-microbe interactions, but allow a basic understanding of how immune homeostasis is regulated in the GIT.

1.3 Bacterial diversity and function in the gastrointestinal tract

1.3.1 Diversity of the gut microbiota

The advent of molecular techniques has enabled the analysis of the GM at unprecedented scales in terms of diversity, dynamics and function, and has revealed the GIT ecosystem to be more complex than previously thought (Bohannan and Hughes, 2003).

The GM is known to have little diversity at higher taxonomic levels (e.g.: phyla) which increases dramatically at lower taxonomical scales. Humans are associated with six to eight bacterial phyla, the Bacteroidetes and Firmicutes (which constitute >90-96% of the microbiome) (Mariat et al., 2009) and to a lesser extent Actinobacteria, Verrucomicrobia, Proteobacteria, Fusobacteria, Spirochaetes and Lentisphaerae (Zoetendal et al., 2008, Rajilić-Stojanović et al., 2007). It is estimated that 500-1,500

bacterial species are associated with the human GM (Eckburg et al., 2005, Qin et al., 2010). While the stomach and small intestines are colonized by simpler bacterial populations, the large intestine of each individual is associated with at least 160 species (Qin et al., 2010), and much greater number of strains.

The human GM is highly variable across individuals (β-diversity) at the lower taxonomic levels (Zoetendal et al., 2001, Stewart et al., 2005). Despite this marked inter-individual variation, a bacterial phylogenetic core across humans has been identified. It includes between 60 and 90 bacterial species (Tap et al., 2009, Qin et al., 2010, Willing et al., 2010), and it is estimated to represent over 40% of an individual's microbiota in terms of abundance (Jalanka-Tuovinen et al., 2011). Additionally, it has been recently proposed that although individuality across humans remains a valid concept, three distinct microbiome structures (enterotypes) are distinguishable among humans, each determined by one abundant bacterial genus (*Ruminococcus*, *Prevotella*, or *Bacteroides*). However, the notion of discrete enterotypes has been questioned, and instead enterotypes have been shown to follow a gradient (Wu et al., 2011). The identification of a common bacterial core, and the possible existence of enterotypes have important consequences to our conception of the GIT ecosystem, as they could indicate the existence of keystone species necessary for community assembly and maintenance. Moreover, functional redundancy in the GIT has been proposed, suggesting that although we might harbor different species, bacterial functions are shared across individuals (Turnbaugh et al., 2009).

Some of the key members of the human GM will now be discussed. One of the most dominant phyla in the human GIT is the Bacteroidetes, which is mainly comprised of members of the *Bacteroides* and *Prevotella* genera, with at least twenty-five species of this phylum being identified as core members of the GM (Gerritsen et al., 2011, Willing et al., 2010). The Bacteroidetes become particularly established in the GIT

with the incorporation of solid foods to the infant diet (Koenig et al., 2011). The other dominant bacterial phylum associated to the GIT, the Firmicutes, is mainly constituted by bacteria in the Clostridia clusters XIVa and IV, which are dominated by the genera *Clostridium*, *Eubacterium*, and *Ruminococcus*. Approximately fifty-five species of this phylum have been identified to be present in over 50% of humans, and thus considered part of the human core (Gerritsen et al., 2011, Willing et al., 2010, Tap et al., 2009). The Actinobacteria are a minor phylum in adulthood, and *Bifidobacterium adolescentis*, *Collinsella aerofaciens* and *Bifidobacterium longum* have been determined to be core human species (Willing et al., 2010, Tap et al., 2009). Archeal species in the GIT are not numerically dominant but are prevalent across humans and have important implications for gut the ecosystem (Dridi et al., 2009). The two most dominant archeal species are *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (Dridi et al., 2009, Gill et al., 2006, Mihajlovski et al., 2008). These methanogenic organisms can convert hydrogen, carbon dioxide and formamate into methane, constituting an additional electron sink in the GIT food-web, and allow further energy harvest from nutrients (Samuel et al., 2007).

1.3.2 Traits of bacteria that colonize the large intestine: The gut microbiota is enzymatically equipped to forage dietary and host derived glycans

The capacity of the GM to derive energy from undigestible dietary compounds is possible through the great enzymatic repertoire of gut microbes that allow them to degrade a wide array of substrates. Different members of the GM have distinct substrate utilization strategies that enable them to occupy the available niches. Some bacteria are considered generalists, they can ferment a wide diversity of substrates

thanks to possessing a large repertoire of genes that encode enzymes involved in substrate utilization. This is the case of the symbiont *Bacteroides thetaiotaomicron*, one of the best described gut organisms in terms of carbohydrate. This organism is one of the core species of the human GIT (Qin et al., 2010), and its vast enzymatic potential for carbohydrate degradation have made it a model organism to study carbohydrate utilization in the GIT. *Bacteroides thetaiotaomicron* has 163 SusC and SusD paralogs (enzymes that bind and import starch), 226 predicted glycolytic enzymes and 15 polysaccharide lyases (Sonnenburg et al., 2005). The opposite strategy is held by microbes that specialize on the utilization of specific substrates, as they possess fewer genes that encode for substrate utilization (Ze et al., 2012). *Ruminococcus bromii* has been identified to be a specialist in starch degradation in the GIT (Ze et al., 2012). *Parabacterides distasonis* is also considered a specialist, and has the enzymatic capability to degrade host glycans, and superior capacity to degrade pectin (Xu et al., 2007). However, not all bacteria in the large intestine are equipped to utilize a large range of complex carbohydrates, but instead rely on shorter carbohydrates released by other bacteria or fermentation end-products. Methanogens for example rely on by-products of bacterial fermentation (H_2, acetate, formate and CO_2) for their growth (Stecher and Hardt, 2008), which allows them to indirectly subsist on a wide range of dietary substrates. Specialists and generalists play differential roles in the complex food web of the GIT (Ze et al., 2012). Specialists are usually the primary degraders of the undigested nutrients and are keystone species for utilization of substrates in the GIT. Generalists can maintain stable populations with a wider range of substrates (Xu et al., 2007).

Expression of gene clusters that encode proteins involved in carbohydrate utilization is tightly regulated in response to nutrient availability, and allow the gut bacteria to adapt to the ever changing substrate sources in the GIT (Sonnenburg et al., 2010).

Members of the Bacteroidetes and the Firmicutes have been shown to regulate gene expression according to the substrates present in the environment (Sonnenburg et al., 2006, Scott et al., 2011b). Moreover, *Bacteroides thetaiotaomicron* can further adapt its metabolism to forage host-derived glycans when deprived of dietary carbohydrates (Sonnenburg et al., 2005).

Trophic and syntrophic interactions in the GIT generate complex food webs among GM members (Stecher and Hardt, 2008). Primary degraders can utilize the NDC and release oligo and monosaccharides that can be utilized by secondary degraders. This seemingly inefficient substrate utilization is key to maintain diversity in the GIT, as more organisms can benefit from NDC. Moreover, primary and secondary degraders can generate SCFAs (acetate, butyrate, propionate, succinate, lactate and formate) and gases such as CO_2 and H_2 (Stecher and Hardt, 2008), that can serve as substrates for other bacteria. A schematic representation of these processes is presented in Figure 1.1. The versatility of enzymes synthesized by gut commensals to utilize diverse carbohydrates, coupled with their ability to sense the nutritional environment and adapt to it, do not only constitute a fitness advantage for the organisms, but have important consequences in the ecology of the GIT, as they enable persistence and stability in this ecosystem.

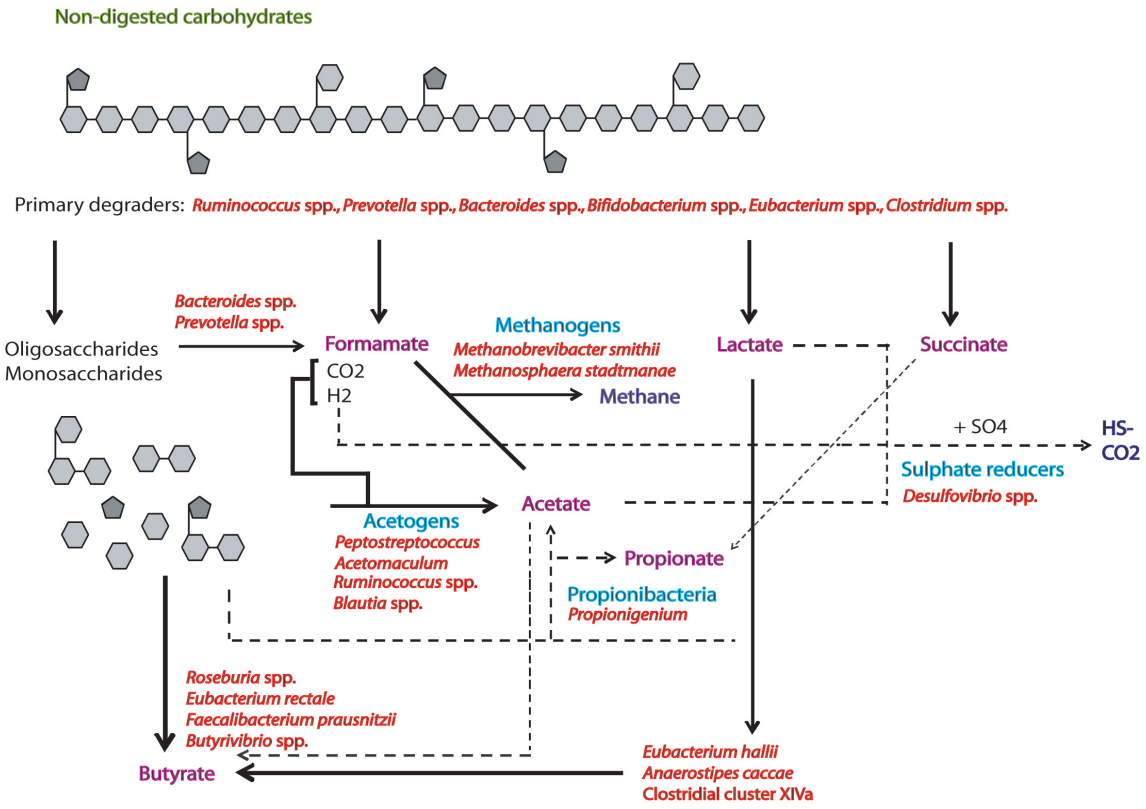

Figure 1.1: **Food webs in the gastrointestinal tract.** Trophic interactions in the gastrointestinal tract between members of the gut microbiota.

1.4 Ecology of the gastrointestinal tract

1.4.1 Characteristics of gut ecosystems: stability, resilience to perturbations, colonization resitance, and individuality

As described above, the GIT is colonized with highly adapted microbes that possess specialized traits to succeed in the gut, and a phylogenetic core comprised of 60-90 phylotypes has been identified. These organisms occupy available niches, which preclude the establishment of incoming organisms due to competitive exclusion, an

important trait that characterizes the GIT (Hardin, 1960, Kassen and Rainew, 2004). The GIT is a dynamic ecosystem, continuously exposed to exogenous (i.e.: diet, antibiotics) and endogenous (i.e.: bile and gastric acid) factors that greatly condition the GM composition. However, the gut ecosystem is remarkably resilient and resistant to environmental disturbances, most GM alterations are temporary and the bacterial community returns to pre-treatment conditions shortly after treatment cessation (Davis et al., 2011, Wu et al., 2011, Dethlefsen et al., 2008, McFarland, 1998, Jernberg et al., 2010). Homeostatic forces make the GM a stable ecosystem, which is another important feature of the GIT (Franks et al., 1998, Vanhoutte et al., 2004, Zoetendal et al., 1998, Jalanka-Tuovinen et al., 2011),

Despite the presence of an autochthonous core, the human gut microbiota is characterized by a high degree of individuality in membership and community structure (β-diversity) (Eckburg et al., 2005, Turnbaugh et al., 2009). The factors driving β-diversity are still not completely understood. Humans are born with a sterile GIT that is successively colonized with dense microbial populations until climax, adult-like communities stabilize (Koenig et al., 2011). This process appears to be 'chaotic', due to a lack of common temporal patterns of diversity between individuals (Palmer et al., 2007), but the process nevertheless results in remarkably stable climax communities. What are the host and environmental factors that favor personalized populations in the gut, and how are the niche environments specifically created or otherwise influenced by host, microbial, environmental and stochastic factors?

1.4.2 Ecological factors that govern the gut microbiome assembly

The most current theory in community ecology includes three perspectives by which communities assemble: (i) Deterministic niche-related, (ii) neutral, and (iii) historic processes (Cavender-Bares et al., 2009, Emerson and Gillespie, 2008). It is likely that all three factors are involved in shaping the assembly of the gut microbiota, but recent observations suggest that their relative importance may differ. The first view of community assembly asserts that communities form according to niche-related, deterministic processes guided by local 'environmental filters (Cavender-Bares et al., 2009, Emerson and Gillespie, 2008). Such processes select for members that possess specific traits that allow competitive exclusion of other members competing for resources (Hardin, 1960, Kassen and Rainew, 2004). There is strong empirical evidence or the importance of deterministic forces in the assembly of the gut microbiota, which might be exerted through such factors as gut physiology, immune responses, and diet (Ley et al., 2008, Ochman et al., 2010, Rawls et al., 2006). Using quantitative genetics, we have recently shown that host genetic factors have a clear measurable contribution to the relative abundance of bacterial genera (Benson et al., 2010). However, host genetic factors were shown to account for only 26% of the variation between individual mouse gut microbiotas, leaving a substantial portion to be explained by other factors. Similarly, although the microbiotas of genetically identical human monozygotic twins are statistically more similar than those of unrelated individuals, they are still remarkably different, and the microbiotas of dizygotic twin pairs are almost as similar (Turnbaugh et al., 2009). These findings indicate that environmental and stochastic factors impact colonization history (Dethlefsen et al., 2006).

Neutral Factors are unlikely to significantly contribute to β-diversity among indi-

viduals. The neutral model for community assembly does not appear to predict community composition in fecal samples (Sloan et al., 2006), and the assumptions made by the Neutral Theory that ecological communities are open to additional colonists, continuously changing, and have non-equilibrium assemblages of species (Cavender-Bares et al., 2009, Emerson and Gillespie, 2008) are in clear contradiction to microbial communities in the human gut, which are temporally stable, resilient to perturbations, and resistant to colonization (Costello et al., 2009, Dethlefsen et al., 2008, Stecher and Hardt, 2008). However, although Neutral Processes are probably negligible, unpredictable and stochastic events are nevertheless important as they do impact colonization history (Dethlefsen et al., 2006).

The third model of community assembly emphasizes the role of historical factors in how communities assemble (Cavender-Bares et al., 2009). In this view, historical patterns of dispersal, which can only be described in probabilistic terms, influence the interactions within the community and matter more than environmental filters. Especially differences in immigration order will affect community assembly as organisms alter niches for themselves and other organisms, thereby shaping the physiochemical properties of the habitat and species interactions. Importantly, Benson and co-workers observed several sets of taxa that were highly positively correlated across the nearly 700 mice (Benson et al., 2010). Collectively, these positive correlations imply that these taxa co-assemble into the microbiota, which then begs the question of how the microbiota would configure if one or more of these organisms were absent. The historical perspective of community assembly opens the door for an evolutionary perspective of community ecology (Cavender-Bares et al., 2009, Emerson and Gillespie, 2008). It challenges the classical assumption in ecology that the traits of the members within a community are static over ecological time-scales, and that communities assemble mainly by ecological fitting in which new members that evolved

elsewhere fit themselves into the open spots within a community. In contrast, both old residents and new arrivals can adapt to niches that are open or newly arise during community assembly, a process referred to as in situ evolution (Cavender-Bares et al., 2009). Bacteria have many unique features that favor rapid evolution over ecological time-scales: short generation times, high population levels, rapid mutation rates, phenotypic plasticity, and high levels of gene flow. Therefore, colonization order and history and in situ evolution could have a central role in the establishment of the human gut microbiota.

Many characteristics of gut microbiota can be explained when we assume a combination of niche-related and historical processes during community assembly. For instance, unpredictable events during assembly would result in differences in community structure in individuals, while in situ evolution would ensure highly adapted phenotypes even in the absence of reliable modes of transmission. Gene flow within members of the community would lead to phenotypic plasticity and functional redundancy among microbial lineages, with the consequence that microbiomes in adult humans are variable in composition but conserved in functional traits (Turnbaugh et al., 2009).

1.4.3 Environmental factors that are likely to impact assembly of the gut microbiota

Given the importance of the microbiota for host physiology and function, the impact of colonization history and immigration order during community assembly is highly relevant as these factors are likely to affect the final composition and functionality of the microbiota, with consequences for health.

Several factors associated with a Westernized lifestyle have been identified to affect

the composition of the gut microbiota. Importantly, these factors are likely to introduce hurdles to symbiont transmission and disrupt community assembly in the GIT. Mode of delivery is the first aspect that conditions the GM composition and assembly (Dominguez-Bello et al., 2010), and is highly relevant as nowadays a large percentage of infants are born by caesarean sections. The neonate is born sterile, and bacterial colonization starts immediately after birth (Koenig et al., 2011). During the passage through the birth canal the neonate becomes associated to the gut microbiota present in this environment. Caesarean section born babies cannot acquire these microbes and instead, are exposed to mostly environmental and probably skin associated microbes from people in contact with the new born, including non-maternal sources (Dominguez-Bello et al., 2010). Caesarean section born babies have been reported to have a delayed colonization of important GM members such as bifidobacteria, lactobacilli and *Bacteroides* (Grönlund et al., 1999).

The feeding regime at the lactation period is another factor of significant importance in the establishment of the GM early in life. Differences in the GM composition have been observed between breast-fed and non-breast-fed infants (Roger et al., 2010, Fallani et al., 2010, Palmer et al., 2007). In addition, the use of antibiotics can cause severe disruptions in the GM assembly process (Dethlefsen et al., 2008, Cotter et al., 2012).

Interestingly, epidemiological studies have linked several environmental factors that are likely to affect gut microbiota assembly to many chronic human diseases with an metabolic or immunological etiology (Bach, 2002). For example, cesarean sections and early administration of antibiotics have been linked to higher rates of allergies, autoimmundiseases, and obesity later in life (Kozyrskyj et al., 2007, Marra et al., 2009, Verhulst et al., 2008, Penders et al., 2007, Bager et al., 2008, Negele et al., 2004). In addition, several studies have revealed that breast feeding prevents the development

of future atopic and allergic diseases, and that these events are related to an altered microbiota (Penders et al., 2007, Wang et al., 2008, Sjögren et al., 2009). Breast feeding has also been associated with decreased risk of childhood obesity (Kalliomäki et al., 2008).

Because these diseases are associated with modern lifestyle habits, and their occurrence is less in developing countries, they are often referred to as Western diseases (Graham-Rowe, 2011). It is striking that virtually all these diseases have been recently linked to the gut microbiota, which led to the intriguing hypothesis (microbiota hypotheisis) that many chronic human diseases might be caused by a disruption of the symbiosis between humans and their gut microbiota (Noverr and Huffnagle, 2004, Blaser and Falkow, 2009). This hypothesis suggests that lifestyle in industrialized societies has introduced profound changes to the human environment (e.g., diet, antibiotics, hospital deliveries, hygiene, etc.) from the conditions to which we have evolved to and that are likely to have occurred too abruptly for the human microbiome to adjust to them. Most importantly, all of these factors impair the transmission of bacterial symbionts and are therefore likely to impact community assembly resulting in altered gut microbiota composition throughout life. Such aberrations of the gut microbiota induced through lifestyle factors could be relevant to the etiology of several complex human diseases whose occurrence has markedly increased in developed countries.

Given these associations, it is essential that we develop a mechanistic understanding of intestinal microbial ecology, and identify bacterial populations in the gut that are associated with diseases. In the coming sections, the association between the gut microbiota and disease will be discussed.

1.5 The gut microbiota in disease

In recent years the GM has been implicated in the etiology of several complex human diseases, mostly with an immunological or metabolic etiology. Diseases that are now associated with the microbiome include inflammatory bowel diseases (IBD), type 1 diabetes (T1D), rheumatoid arthritis (RA), multiple sclerosis (MS), dyslipidemia, insulin resistance, type 2 diabetes (T2D), colon cancer, allergies, coronary heart disease (CHD) and obesity (Berer and Krishnamoorthy, 2012, Bäckhed, 2011). Host genetic factors have been identified in the predisposition to disease, but do not completely explain disease manifestation.

The evidence for a contribution of the gut microbiota to Western diseases is three fold. First, virtually all the environmental changes associated with Western diseases (C-sections, antibiotics early in life, formula feeding, clean living conditions, low fiber foods, etc.) are likely to disrupt assembly of the microbiota, as they prevent efficient symbiont transmission (see above). Second, animal models are often protected from disease when animals remain GF. Third, aberrations in the gut microbiota are associated with virtually all of these diseases, both in humans and in animal models. Table 1.1 summarizes the diseases that have been associated with the gut microbiota, and some of the main findings that link pathophysiology with the gut microbiome. Because of the scope of the research presented in this thesis, this discussion will focus on the importance of the gut microbiota in obesity and related metabolic disorders (type II diabetes, CHD).

Although the diseases associated with the gut microbiome show very different etiologies, there seems to be a common link between them: the interaction of the microbiome with the host immune system. As mentioned, the gut microbiota is important for the correct development of host immune functions (Graham-Rowe, 2011). The ab-

sence of specific microbes in early life, coupled with a genetic predisposition to disease, might cause dysfunctional immune reactions that underlie disease (Berer and Krishnamoorthy, 2012, Graham-Rowe, 2011). In addition, the assembly of a microbiome enriched in microbes that induce inflammation and/or other pathologic immune reactions (e.g. induction of Th17 response) might contribute to disease (Atarashi and Honda, 2011). Moreover, the interrelationship between the microbiota and the immune system might also underlie diseases such as colon cancer or metabolic disorders. Inflammation is a major early contributor to the development of colon cancer, and it is likely caused through gut bacteria (Rubin et al., 2012, Carbonero et al., 2012). In addition, obesity and related metabolic disorders (e.g. insulin resistance) are associated with what is referred to as a 'metabolic inflammation', and there is strong evidence that this inflammatory response is not only caused by gut microbes but also has a causative role in pathophysiology (Kern et al., 2001, Gregor and Hotamisligil, 2011, Lee et al., 2009).

1.5.1 A role of the gut microbiota in obesity

1.5.1.1 The impact of the gut microbiota in the host's energy metabolism

The contribution of GM to host energy metabolism extends beyond energy provision to the host from undigested dietary nutrients, but is also manifested through their regulation of several metabolic pathways that induce energy storage in the host.
SCFAs signaling of G-coupled receptors GPR41 and GPR43 modulate host energy metabolism (Murphy et al., 2010). GPR41 is involved in the production of several gut peptides, peptide YY (PYY), ghrelin and glucagon-like peptide 1 (GLP1), which are involved in the modulation of insulin secretion, the host's lipidome, glu-

cose metabolism and food intake (Cani et al., 2007, Samuel et al., 2008). Signaling of GPR43 by acetate has been associated to improved immune capacity of the host to prevent infection by enteropathogenic *Escherichia coli*, which has been attributed to promote enhanced gut barrier epithelial function (Maslowski et al., 2009, Fukuda et al., 2011).

Bile acid represents another example of diet-host-microbiome interactions. Primary bile acids (BA), cholic and chenodeoxicholic acids, are synthesized in the liver from cholesterol. Their amphipathic nature allows them to emulsify the dietary lipids in the small intestine to facilitate their absorption. Bacteria can further process primary bile acids through deconjugation and dehydroxylation reactions, generating secondary bile acids (Ridlon et al., 2006). These modifications alter the hydrophobicity of BA with consequences to their lipid solubilization capacity and absorption, and condition their metabolic fate (Nicholson et al., 2004). Moreover, the gut microbiota composition can significantly impact the BA pool, consequently affecting the host lipidome (Martin et al., 2007, Velagapudi et al., 2010). Microbiota generated lithocholic acid has been shown to activate G-coupled receptor TGR5, which induces GLP-1 secretion in colonocytes affecting pancreatic function and glucose metabolism (Thomas et al., 2008, Reimann et al., 2008). TGR5 has futher effects on brown adipose tissue where it increases energy expenditure through production of triiodothyronine (Watanabe et al., 2006). Conversely, bile acids can modulate the GM composition (Islam et al., 2011).

There are additional mechanisms through which the gut microbiota affects the host's energy metabolism. GF animal experiments have revealed that the association of the host with the GM suppresses the expression of angiopoietin-like protein 4 (Angptl4), a strong repressor of lipoprotein lipase (LPL) therefore increasing adiposity in the host (Bäckhed et al., 2004). Apart from suppressing LPL activity, Angptl4 has also

been suggested to regulate fatty acid oxidation in the skeletal muscle by affecting the expression of carnitine palmitoyl transferase-1 (Cpt-1) and medium-chain acyl-CoA dehydrogenase (Bäckhed et al., 2004). The GM has been further implicated in the downregulation of AMP-activated protein kinase (AMPK) in hepatic cells and in skeletal muscle (Bäckhed et al., 2007). AMPK is an enzyme implicated in host cellular energy homeostasis. AMPK can sense the cellular energy state and inhibit anabolic pathways while stimulating catabolic ones in conditions of energy depravation. The presence of the GM downregulates AMPK activity, causing the host to increase its energy storage (Bäckhed et al., 2007). Increased glucose uptake in the small intestine has also been observed in microbiota-associated mice, and although the mechanisms by which this takes place are not known, it contributes to dietary energy harvest (Bäckhed et al., 2004).

The ability of the GM to ferment non-digested nutrients that would otherwise represent wasted energy, allow the microorganisms to harvest energy for their own growth and survival, and also provide the host with additional energy sources. The above mentioned mechanisms illustrate diverse forms in which the GM impacts the host energy metabolism that extend beyond increased energy harvest. This increased capacity for energy harvest and storage could have once been a beneficial trait conferred by the gut microbiota for our ancestors when food was scarce and not as readily available as today. The large food supply and the lack of physical exercise characteristic of Westernized societies have turned this into a disadvantage.

1.5.1.2 Associations between the gut microbiome and obesity

In the past 50 years 'Westernized' societies have experienced a dramatic rise in the prevalence of obesity (Gregor and Hotamisligil, 2011). Both genetic predisposition and an excessive caloric intake contribute to the manifestation of obesity (Cani and

Delzenne, 2009). The fact that GF animals have been shown to be protected from developing obesity and insulin resistance (Bäckhed et al., 2004, Caesar et al., 2012), coupled with observations indicating that conventionally raised animals have 40% more adipocity than their GF counterparts despite a lower feed intake (Bäckhed et al., 2004) have prompted research to investigate the links between the GM, obesity and metabolic aberrations. It must be noted that some studies have shown contradicting results as to the contribution of the GM to obesity. Fleissner and co-workers (2010) concluded that GF mice were not protected from diet-induced obesity, and undermined the role of Angplt4 as a host-microbe mediator of fat storage. Nevertheless, GM transplantations from obese to GF animals have indicated that the obese phenotype can be transferred (Turnbaugh et al., 2008, 2006, Vijay-Kumar et al., 2010). A further link between obesity and the GM was suggested through a genetic mouse model with impaired sensing of GM commensals. Toll-like receptors (TLRs) are pathogen-sensing receptors of the innate immune system (Vijay-Kumar et al., 2010). In this study, researchers showed that Tlr5 deficient mice have an altered GM composition, and that transplantation of $Tlr5^{-/-}$ GIT contents to GF mice transferred milder features of the metabolic aberrations and obesity, which the researchers concluded could be attributed to an altered GM composition.

Further associations between the GM and obesity have stemmed from observations that ascribe a dysbiotic microbial phenotype to obesity (Table 1.1). First, obese individuals have been shown to harbor a less diverse microbiota than normoweight individuals (Turnbaugh et al., 2009). In addition, both animal experiments and comparisons between humans have revealed that obesity is associated with a decrease in the abundance of the phylum Bacteroidetes (Ley et al., 2005, 2006). These shifts were linked to an increased dietary energy harvest capacity with increases in the Firmicutes/Bacteroidetes ratio (Turnbaugh et al., 2006), and suggested that this bacterial dysbiosis can cause obesity. However, the importance of dysbiosis in obesity has been questioned. Increased abundance of Bacteroidetes has not been detected in several human studies. In addition, Bacteroidetes did not correlate with body mass index (BMI) (Duncan et al., 2008, Schwiertz et al., 2010). A possible explanation to these conflicting results is that Bacteroidetes abundance may not be conditioned by obesity

but by energy intake and diet (Furet et al., 2010, Ley et al., 2006). Employment of different methodologies and differences between the human cohorts among studies may also explain the differences encountered between studies (Flint, 2011).

Although the importance of dysbiosis is unclear, the microbiota clearly contributes to host nutrient utilization. This trait was likely shaped during co-evolution and beneficial throughout most of human evolution when diet was limited. An increased capacity for energy harvest and storage could have once been a beneficial trait conferred by the gut microbiota for our ancestors when food was scarce and not as readily available as today. However, the large reliable food supplies of often energy dense foods and the lack of physical exercise characteristic of Westernized societies have now turned this once mutualistic trait of our bacterial symbionts into something many would prefer to avoid.

Table 1.1: **Dysbiosis in microbiota associated diseases.**

Pathology	Study population	Main findings	Bibliography
Obesity		Dysbiosis in obese versus normoweight	
	12 Obese 2 Normoweight	↑ Bacteroidetes ↓ Firmicutes	(Ley et al., 2006)
	23 Obese/overweight 14 Normoweight	↓ butyrate producing Clostridia cluster XVIa	(Duncan et al., 2008)
	25 Obese/overweight children 24 Normoweight children	Composition at infancy prior to obesity development ↓ Bifidobacteria ↑ *Staphylococcus aureus*	(Kalliomäki et al., 2008)
	20 Obese	↓ Bacteroidetes	(Armougom et al., 2009)

Continued on next page

Table 1.1 – continued from previous page

Pathology	Study population	Main findings	Bibliography
	20 Normoweight	↑ *Lactobacillus*	
	31 Monozygotic twin pairs	↑ Actinobacteria	(Turnbaugh et al., 2009)
	23 Dizygotic twin pairs	↑ Firmicutes	
	46 Mothers of twins	↓ Bacteroidetes	
	3 Obese	↑ Prevotellaceae	(Zhang et al., 2009)
	3 Normoweight	↑ Methanogens	
	3 gastric bypass surgery		
	33 Obese	↑ Bacteroidetes	(Schwiertz et al., 2010)
	35 Overweight	↓ Firmicutes	
	30 Normoweight		
	30 obese	↓ *Bacteroides*/ *Prevotella*	(Furet et al., 2010)
	13 Normoweight		
T2D		Dysbiosis in T2D versus control	
	18 diabetic	↓ Firmicutes	(Larsen et al., 2010)
	18 healthy	↓ Clostridia	
Type 1 diabetes		Dysbiosis in T1D control	
	4 future T1D	↓ Firmicutes	(Giongo et al., 2011)
	4 controls	↑ Bacteroidetes	
Rheumatoid arthritis		Dysbiosis in RA versus control	
	51 RA patients	↓ Bifidobacteria	(Vaahtovuo et al., 2008)

Continued on next page

Table 1.1 – continued from previous page

Pathology	Study population	Main findings	Bibliography
cont.	50 fibromyalgia	↓ *Bacteroides-Porphyromona-Prevotella* ↓ *Eubacterium rectale - Clostridium coccoides* ↓ *Bacteroides fragilis*	(Vaahtovuo et al., 2008)
Inflammatory bowel disease		Dysbiosis in IBD versus control	
	68 CD patients 61 UC patients 61 controls 27/63 CD patients	↑ Enterobacteriaceae ↓ Firmicutes ↓ Bacteroidetes ↓ *Faecalibacterium*	(Frank et al., 2007)
	8 UC patients 16/102 controls	↑ Adherent/invasive *Escherichia coli*	(Darfeuille-Michaud et al., 2004)
	13 ileal CD patients 8 colon CD patients 7 controls	↑ Adherent/invasive *Escherichia coli* ↓ *Faecalibacterium*	(Baumgart et al., 2007)
	10 Twin pairs with CD 8 Healthy twin pairs	↓ *Faecalibacterium* ↓ *Roseburia* ↑ Enterobacteriaceae ↑ *Ruminococcus gnavus*	(Willing et al., 2010)
	68 CD patients (in remission) 84 Unaffected relatives	↓ *Faecalibacterium prausnitzii* ↓ *Dialister invisus*	(Joossens et al., 2011)

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Table 1.1 – continued from previous page

Pathology	Study population	Main findings	Bibliography
	55 controls	↓ *Bifidobacterium adolescentis* ↑ *Ruminococcus gnavus*	
	12 CD patients 6 UC patients 5 controls	↓ Firmicutes IBD ↑ Bacteroidetes IBD ↑ Enterobacteriaceae CD	(Walker et al., 2011b)

1.5.2 The gut microbiota triggers low-grade inflammation associated with obesity and metabolic disorders

Obesity is associated with a cluster of metabolic disorders related to insulin resistance, dyslipidemia and the development of type 2 diabetes and cardiovascular disease (CVD) (Cani and Delzenne, 2009). Obese humans are characterized by increased levels of circulating and adipose derived proinflammatory cytokines and biomarkers of inflammation, including interleukin-6 (IL-6) and tumor necrosis factor-α (Kern et al., 2001). This phenotype has been demonstrated to be reversible with weight loss (Dandona et al., 1998), and to contribute to the development of the obese related pathologies (Gregor and Hotamisligil, 2011, Lee et al., 2009). A contribution of the GM to the development of low-grade inflammation has been determined (Cani et al., 2007). High-fat (HF) diet induced obesity animal models have been employed to investigate the relation between diet-microbiome-host in their connection to obesity. It was determined that a HF diet in association with the GM can trigger inflammation, and that the inflammatory response of the host antecedes the onset of obesity

and insulin resistance, as TNF-α levels were significantly increased before weight gain or insulin insensitivity was observed (Ding et al., 2010). Animal experiments based on this model conducted by Cani and co-workers (2007) determined, first, that lipopolysaccharides (LPS) induced metabolic endotoxemia. LPS are molecules present in the outer membrane of Gram-negative bacteria that serve as recognition factors by the host immune system and can act as endotoxins and induce pronounced immune responses in the host. Second, the researchers demonstrated that continuous subcutaneous infusion of bacterial LPS caused weight gain and insulin resistance, independent of a HF diet. Another piece of evidence supporting for a GM induced low-grade inflammation, is that antibiotic treated obese mice presented lowered LPS and TNF-α expression, weight loss and an improved glycemic profile (Cani et al., 2008, Membrez et al., 2008). Recent research has indicated that bacterial LPS instigates accumulation of macrophages in white adipose tissue, but not obesity or impairment of the glucose metabolism (Caesar et al., 2012).

The increased plasma levels of LPS linked to HF diets have been attributed to chylomicron-facilitated absorption of LPS enhanced by increased ingestion of fatty acids (Caesar et al., 2010). An alternative or complementary mechanism through which plasma LPS is thought to be increased, is by the paracellular transport of LPS through tight junctions of the intestinal epithelium (Caesar et al., 2010). Decreased levels and increased interspersion of tight junctions proteins occludin and ZO-1 have been reported in obese mice (Cani et al., 2009).

These studies depict the link between obesity and the gut microbiota as highly complex. Moreover, despite connections between GM and obesity being identified that suggest the causation of metabolic disorders by gut bacteria, the extent of the contribution of the GM to the development of these diseases remains unclear.

1.5.3 Implication of the gut microbiota in metabolic disorders and cardiovascular disease

Obesity is associated with an increased risk of cardiovascular disease (CVD) (Gregor and Hotamisligil, 2011). As mentioned above, several lines of research have evidenced a contribution of the GM to obesity, thus generating a higher risk of CVD events. Interestingly, the connection between the GM and CVD extends beyond the link between obesity and CVD. Dietary phosphatidylcholine has been determined to increase the risk of CVD with the GM as a mediator (Wang et al., 2011). Choline is released from phosphatidylcholine by host enzymes. The GM further processes choline into trimethylamine (TMA), which is absorbed by the host and converted into trimethylamine N-oxide (TMAO). Wang and colleagues (2011) demonstrated that bacterial action on choline directly impact plasma TMAO concentrations. In atherosclerotic prone animals TMAO is associated with arterial plaque development and increased risk for CVD, which can be reversed upon treatment of the animals with antibiotics. A study conducted in humans did not detect differences in the GM composition of atherosclerotic patients compared to healthy subjects (Koren et al., 2011), suggesting that choline metabolism into TMA is an ubiquitous trait of the GM, and that no specific bacterial population is associated with it. However, supplementation of specific bacteria (*Lactobacillus rhamnosus* and *Lactobacillus paracasei*) to humanized mouse models decreased hepatic TMAO levels, suggesting that specific bacteria can impact host-choline metabolism.

To further illustrate the complexities between microbe-host interactions, current research has highlighted the involvement of the GM on yet another metabolic disorder, non-alcoholic fatty liver disease (NAFLD). NAFLD is a metabolic liver condition associated with obesity and insulin resistance. This asymptomatic state can develop

into non-alcoholic steatohepatitis (NASH), which can lead to cirrhosis. The mechanisms by which the GM was linked to NAFLD and NASH involve the inflammasome. The inflammasome is a multiprotein oligomer that is expressed in myeloid cells as part of the innate immune system, and can take several configurations depending on the activator that initiates its assembly. Upon activation, the inflammasome triggers the maturation of inflammatory cytokines IL-18 and IL-1β. Inflammasomes can sense and regulate GM composition. Henao-Mejia and co-workers (2012) established, first, that genetic mice lacking functional inflammasomes harbored a dysbiotic GM. Second, that co-housing of inflammasome impaired mice ($Asc^{-/-}$, $Casp1^{-/-}$, $IL\text{-}18^{-/-}$ or $Nlrp3^{-/-}$) with wild type mice resulted in the transmission of the NASH phenotype to the wild type mice to a comparable extent. Next, the authors established that TLR knockout mice were resistant to NASH development due to co-housing with inflammasome impaired animals, indicating the sensing of microbial factors is needed for the progression of disease.

Dysbiosis has also been observed in individuals with type-2 diabetes (T2D). T2D patients have been reported to have increased abundance of Bacilli and Betaproteobacteria, and decreased proportions of Clostridia (Larsen et al., 2010).

1.5.4 The importance of dysbiosis

Although GF animal experiments have provided strong evidence indicating that the GM is involved in the etiology of diseases, the importance of dysbiosis is often much less clear. Most importantly, the directionality of the association between dysbiosis and disease has not been elucidated. As described above, the dysbiosis associated with obesity might be caused through the differences in the dietary habits between obese and lean subjects. In addition, IBD causes similar aberrations in the gut micro-

biome (e.g. increased Enterobacteriacea) than acute inflammation or inflammation associated with other diseases (for example transplant rejection (Oh et al., 2012), suggesting that some features of dysbiosis are a response and not a cause of inflammation. It is possible that the physiological and immunological changes associated with metabolic disorders related to obesity affect the GM composition, but this is much less understood. In this thesis (Chapter 5), evidence is presented that indicates that the host cholesterol metabolism shapes the composition of the gut microbiota.

1.6 Impact of dietary habits on the gut microbiota in relation to health

1.6.1 Effects of dietary habits on the gut microbiome

1.6.2 Dietary regimes in early infancy

Diet is a primary factor that shapes GM composition (Flint, 2011). Human milk contains over two hundred different oligosaccharides (Zivkovic et al., 2011) that provide nourishment to the infant and the GM (Harmsen et al., 2000). Human milk oligosaccharides especially support the growth of *Bifidobacterium* spp. and *Bacteroides* spp. (Marcobal et al., 2010, Xiao et al., 2010). The dietary regime at the lactation period determines differences in GM composition between breast-fed and formula-fed infants. Non-breast fed infants are characterized by a decreased abundance in bifidobacteria, the association with different bifidobacteria species than breast-fed infants, and have increased proportions of *Bacteroides*, Clostridia and Proteobacteria (Roger et al., 2010, Fallani et al., 2010).

The most striking example of the impact of diet on the GM composition is weaning

and incorporation of solid foods to an infants' diet, which drive the most dramatic alterations in the gut bacterial community that healthy individuals experience in their lifetime. During this period, the GIT environment is exposed to 'novel' carbohydrates that induce marked changes in GM structure, especially a decline in the abundance of bifidobacteria and an expansion of Bacteroidetes (Koenig et al., 2011).

1.6.3 Diets high in non-digestible carbohydrates, fat or protein have differential impacts on the gut microbiota

The impact of dietary habits on microbiome structure has been indirectly provided by studies investigating the GM composition of individuals living in Westernized versus non-Westernized societies. Yatsunenko and co-workers (2012), compared the gut microbiome of people from Malawi, US metropolitan areas and Venezuelan Amerindians, while De Filippo and colleagues (2010) investigated the GM of children in Burkina Faso and Europe. These studies revealed important differences in the conformation and function of the GM. Notably, although genetic, cultural, geographical and lifestyle factors are bound to impact the gut microbiome and differentiate GM composition, both studies identified diet as a key feature driving the differences observed. An overrepresentation of *Xylanibacter* (a xylan and cellulose degrading organism) was observed in Burkina Faso children, while this genus was undetected in European children (De Filippo et al., 2010). In addition, SCFAs concentrations were significantly higher in the feces of the African children, evidencing a greater carbohydrate intake and metabolism in the GIT (De Filippo et al., 2010). Yatsunenko and co-workers (2012) determined that US gut metagenomes were enriched in catabolic enzymes of simple sugars, sugar substitutes and host glycans, whereas Malawians and Amerindi-

ans who have a cassava and maize rich diet had an abundance of α-amylase encoding genes (Yatsunenko et al., 2012). In addition, genes encoding for glutamate synthase, characteristic of herbivores, were overrepresented in Malawians and Amerindians, while US individuals had an overabundance of glutamine degrading enzymes, a trait of carnivores (Muegge et al., 2011). Also, the presence of enzymes implicated in the bile acid metabolism was greater in US microbiomes, potentially reflecting higher lipid consumption in Western diets (Yatsunenko et al., 2012). Interestingly, a point of convergence between the studies was the higher abundance of *Prevotella* in individuals of Malawi, Burkina Faso and the Venezuelan Amerindians compared to Westerners. The rumen associated *Prevotella ruminocolla* has been described to possess particular xylanase activity (Kabel et al., 2011). A genome search using the Integrated Microbial Genomes (IMG) platform of the Joint Genome Institute (JGI) revealed that the human gut commensal *Prevotella copri* has two β-1,4-xylanases and a predictive xylanase encoding genes, two pectinmethylesterases and five endoglucanases encoding mainly for cellulase activity. These findings portray the aptitude of *Prevotella* as primary carbohydrate degraders, and help explain why this organism is more abundant in diets rich in complex carbohydrates. Concordantly, a recent study found that long-term carbohydrate rich diets were associated with higher *Prevotella* proportions in the GIT and favored the *Prevotella*-dominant enterotype (Wu et al., 2011).

Several studies have documented that not only carbohydrates but also dietary proteins and lipids have an effect on the gut microbiome. Most of the knowledge we have on the effect of a HF diet on GM composition has stemmed from animal models of obesity in which a HF-diet is used to develop the obese phenotype. This has caused difficulties in segregating the effects of the HF diet *per se*, and the potential contributions of a higher body fat and inflammation on the microbiome. A few studies have tried to unravel these confoundiing factors, and have concluded that the effect

of a HF diet prevails over the microbial alterations caused by the obese phenotype (Ravussin et al., 2012, Murphy et al., 2010). A HF driven expansion of Firmicutes has been consistently described in several studies (Hildebrandt et al., 2009, Murphy et al., 2010, Ravussin et al., 2012), and members of the Clostridiaceae family were determined to be the most affected (Hildebrandt et al., 2009). Increased abundance of Proteobacteria with HF diets has also been reported (Hildebrandt et al., 2009). Abundance of Bacteroidetes, on the other hand, has been shown to both increase and decrease depending on the study (Hildebrandt et al., 2009, Ravussin et al., 2012). Concomitant with compositional changes of the GM, metabolic changes have also been ascribed to HF diets. Hildebrandt and colleagues (2009) determined that genes involved in amino acid and carbohydrate metabolism decreased in abundance, and those implicated in membrane transport increased. Genes involved in sugar transport have also been determined to be increased in HF diet regimes (Hildebrandt et al., 2009, Turnbaugh et al., 2008).

High protein consumption has also been determined to affect the GM composition and metabolism. Overall, a *Bacteroides* dominated microbiota is associated with protein intake (Wu et al., 2011). In addition, a dietary regime high in protein and low in carbohydrates was identified to cause significant decreases in the *Roseburia/Eubacterium rectale* group concurrent with a decreased proportion of butyrate in the SCFA pool compared to subjects on a high-protein moderate-carbohydrate diet (Russell et al., 2011). However, a higher supplementation of peptides to an NDC-rich medium is associated with increased butyrate production and proportions of *Roseburia/Eubacterium rectale*.

1.6.4 Impact of diet-induced alterations of the gut microbiota on health

As popular wisdom holds: 'We are what we eat'. Epidemiological research has strongly linked dietary habits to disease, and some of these links might be due to the gut microbiota.

The benefits of a low fat diet in terms of obesity and metabolic disorders do not only originate through a decreased fat absorption by the host with consequent reduction in fat deposit, insulin resistance and dyslipidemia, but also stem from a reduction in the systemic inflammation associated with impaired gut barrier function and increase of plasma LPS elicited through dietary fat. High fat diets have been shown to increase permeability of the gut, and higher gut permeability is associated with obesity in humans (Cani et al., 2009).

Another disease that is determined to have the GM at the intersection between diet and disease is colorectal cancer, the fourth most common cause of cancer related death (Jemal et al., 2011). Diet has been determined to be the most important contributing factor to the etiology of this disease, as it is estimated that 80% of colorectal cancers are diet related, and that a diet high in fats and proteins (especially of animal origin) and low in carbohydrates is detrimental (Willett, 1995). The GM has been implicated in the development of colorectal cancer in several ways. First, bacterial fermentation of dietary proteins leads to the formation of toxic compounds such as ammonia, amines and indoles, which have been linked to carcinogenesis (Hughes et al., 2000). Second, NDC ingestion is deemed beneficial for the prevention of this disease as its fermentation by-products, SCFAs, are deemed protective. Butyrate promotes apoptiosis in colonocytes and regulates gene expression (Csordas, 1996, Tazoe et al., 2008, Heerdt et al., 1994). Moreover, bacterial fermentation of plant derived carbohydates

has been shown to release protective phenolic compounds with anti-carcinogenic capacity such as ferulic acid derivatives (Hamer et al., 2008, Russell et al., 2011). For these reasons, diets rich in whole grains and fiber have been included in dietary recommendations, and researchers have questioned the value of long-term diets high in protein and low in complex carbohydrates (Russell et al., 2011).

An additional benefit of NDC in the context of IBD, and particularly Crohn's disease, is the enhanced butyrate concentrations and expansion of *Faecalibacterium prausnitzii* populations given its association to decreased rate of relapse after surgery (Sokol et al., 2008). Despite these promising associations, dietary interventional trials with FOS in CD patients have failed to stimulate *Faecalibacterium prausnitzii* populations in the GIT (Benjamin et al., 2011).

1.7 Dietary strategies to modulate the GM

The interest in the modulation of the gut microbiota to promote human health dates back more than a century when Metchnikoff hypothesized that reducing the number of 'putrefactive' bacteria in the GIT could improve gastrointestinal health and prolong life (Metchnikoff, 1908). The increased understanding of the critical role of the gut microbiota in human health and the aberrations in the GM linked to several complex diseases have intensified the interest in dietary strategies that modulate the gut ecosystem. Many studies have shown that dietary strategies can be used to modulate the GM, suggesting that aberrations or imbalances associated with disease could be corrected and host health improved. These dietary strategies have focused primarily on probiotics and prebiotics, and more recently on synbiotics (a combination of pro- and prebiotics). Probiotics are live microorganisms that when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2001). A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health. Prebiotics are defined as 'selectively

fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health' (Roberfroid, 2007). Several prebiotics are now widely commercially used in foods, including inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS). Moreover, there has been increased interest to use dietary fibers (including resistant starch), other NDCs, and whole grains to modulate the gut microbiota. In this section an overview of the main NDCs used to impact GM composition and the main effects observed in *in vivo* human trials is presented (Table 1.2).

Table 1.2: **Impact of non-digestible carbohydrates in *in vivo* human trials.**

NDC	Number of subjects	Treatment duration	Daily dose	Method	Outcome	Bibliography
FOS						
	8	15 days	15 g	Culture	↑ Bifidobacteria ↓ Clostridia ↓ *Bacteroides* ↓ Fusobacteria	(Gibson and Roberfroid, 1995)
	12	25 days	4 g	Culture	↑ Bifidobacteria	(Buddington RK, 1996)
	8	5 weeks	8 g	FISH	↑ Bifidobacteria	(Menne and Guggenbuhl, 2000)
	40	7 days	2.5-10 g	Culture	↑ Bifidobacteria	(Bouhnik et al., 2006)
Inulin						
	10	9 days	9 g	FISH DGGE	↑ Bifidobacteria ↑ *Eubacterium rectale* group	(Harmsen, 2002)

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Table 1.2 – continued from previous page

NDC	Number of subjects	Treatment duration	Daily dose	Method	Outcome	Bibliography
	39	4 weeks	2.5 g	Culture	↑ Bifidobacteria	(Bouhnik et al., 2007)
	30	2 weeks	5 or 8 g	FISH	↑ Bifidobacteria	(Kolida et al., 2007)
GOS						
	12	7 days	36, 71, 143 mg	Culture	↑ Bifidobacteria	(Ito et al., 1990)
	8	21 days	10 g	Culture	↑ Bifidobacteria	(Bouhnik et al., 1997)
	30	4 weeks	2.4 g	Culture	↑ Bifidobacteria	(Gopal et al., 2003)
	8	1 week	2.5, 5, 7, 10 g	Culture	↑ Bifidobacteria	(Bouhnik et al., 2004)
	18	3 week	2.5, 5, 10 g	DGGE Pyroseq.	↑ Bifidobacteria ↓ *Bacteroides* group	(Davis et al., 2011)
Resistant starch						
	46	4 weeks	22 g	DGGE	↑ *Ruminococcus bromii*	(Abell et al., 2008)
	14	3 weeks	22 g	qPCR Pyroseq.	↑ *Ruminococcus bromii* ↑ *Eubacterium rectale* ↑ *Oscillibacter*	(Walker et al., 2011a)
Arabinoxylans						
	63	3 weeks	3 or10 g	FISH	↑ Bifidobacteria	(François et al., 2012)

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Table 1.2 – continued from previous page

NDC	Number of subjects	Treatment duration	Daily dose	Method	Outcome	Bibliography
	20	3 weeks	10 g	qPCR	↑ Bifidobacteria	(Cloetens et al., 2010)

1.7.0.1 Dietary non-digestible carbohydrates affect the gut microbiota composition and function

One of the main carbohydrates that reach the large intestine are resistant starches. Starches are the main storage carbohydrates present in plants, a fraction of which is denominated resistant starch (RS) in virtue of its resistance to amylases in the small intestine, and can be fermented by bacteria in the large intestine. Over 50% of gut bacterial isolates have the ability to degrade starches *in vitro* (Salyers et al., 1977, Walker et al., 2011a). Despite this, not many bacterial species have been determined to be enriched in *in vivo* dietary human trials with RS. The main species identfied to respond to RS diets are *Eubacterium rectale* and *Ruminococcus bromii* (Abell et al., 2008, Walker et al., 2011a).

Fructans are another type of plant storage carbohydrates, and include inulin and their shorter chain counterparts fructooligosacchrides (FOS). Consumption of inulin has been notable for stimulating bifidobacteria (Scott et al., 2011a). Moreover, inulin and FOS enhance butyrate production in the GIT and butyrate producing species such as *Faecalibacterium prausnitzii* (Ramirez-Farias et al., 2009). Galactooligosaccharides (GOS) are another class of carbohydrate regarded for its bifidogenic potential. GOS were determined to elicit an important bifidogenic response in 50% of individuals and to have a dose-response effect in some persons (Davis et al., 2011).

Another important source of carbohydrates in the large intestine are plant cell wall polysaccharides, which include a wide variety of carbohydrates such as pectin, cellulose and xylans. Most of the bacteria reported to degrade these polysaccharides belong to the Firmicutes phylum. Xylan utilizers include *Roseburia intestinalis* and *Butyrivibrio fibrinisolvens* (Rumney et al., 1995), while *Faecalibacterium prausnitzii* and *Eubacterium eligens* are pecting degraders (Lopez-Siles et al., 2012, Salyers et al., 1977).

1.7.1 Mechanisms by which non-digestible carbohydrates induce specific changes in the gut microbiota

By definition, prebiotics specifically support the growth of a specific bacterial species in the GIT. Clearly, as described above (section 'Traits of the gut microbiota'), substrate specificity exists to some degree within the gut microbiota (i.e.: inulin is utilized by bifiodbacteria and the *Eubacterium rectale* clostridial group). For example, the prebiotic inulin is only utilized by a restricted group of gut microbes. In addition, recent research, including research described in this thesis (Chapter 2), showed that even carbohydrates such as resistant starches, which are utilized by many gut microbes, induce remarkable specific alterations to the gut microbiome. However, it is now generally recognized that most prebiotics are not as selectively fermented as previously proposed. It is therefore evident that more complex competitive and co-operative interactions between the gut microbiome define the effect of NDC on gut microbiota composition.

Cross-feeding in the GIT involves the utilization of partial breakdown products from primary degraders by secondary utilizers, as well as the use of fermentation by-products such as SCFAs. Flint's group has nicely evidenced these co-operative inter-

actions in *in vitro* experiments in which co-culturing of *Ruminococcus bromii* with *Bifidobacterium adolescentis*, *Eubacterium rectale* or *Bacteroides thetaiotaomicron* led not only to an increased utilization of RS2 and RS3 than the respective mono-cultures, but even favored the growth of *Eubacterium rectale* (Ze et al., 2012). Flint and colleagues have also performed *in vitro* assays showcasing the relevance of cross-feeding between bacteria. *Eubacterium halii* and *Anaerostipes caccae* cannot grow on RS in pure culture, but are able to grow if *Bifidobacterium adolescentis* is present in the culture (Belenguer et al., 2006). These syntrophic associations are possible through the utilization of *Bifidobacterium adolescentis*-generated lactate by *Eubacterium halii* and *Anaerostipes caccae*. Moreover, these co-operative networks result in the production of butyrate by *Eubacterium halii* and *Anaerostipes caccae* with positive consequences to host health. Another important by-product of bacterial fermentation implicated in cross-feeding networks is hydrogen. For many bacteria, hydrogen constitutes an end product of fermentation. However, a few members of the GM are specialized in hydrogen utilization as an energy source. Acetogens, methanogens and sulfate reducers can utilize hydrogen produced by other bacteria and add another link to the GIT energy network.

These observations allow us to hypothsize that nutrient utilization in the GIT is determined by the composition of the GM as a whole, and dependent on competitive and co-operative interactions between its members. Moreover, these considerations imply that predicting the modulation of the GM by dietary substrates is a rather difficult task from an *in vivo* context. Further obstacles may arise when one considers the potential effect of the food matrix and the interaction with other nutrients present in the diet in view of predicting modulatory outcomes of the GM by diet.

1.8 Open questions

The evolved dependence of mammals with their GM to ensure its own development suggests the profound importance of the correct establishment of the GM for the host to achieve homeostasis throughout life. Our modern lifestyle has challenged this symbiosis, with consequences to our health. In order to restore this symbiosis, a clear understanding of the mechanisms that control GM assembly and composition need to be understood. Dietary strategies constitute one of the most effective tools to modulate the microbiome composition and function. The work presented in this dissertation focuses on dietary strategies to modulate the GM, their impact on health, and the interrelationship between diet, the microbiome and health.

Animal experiments constitute an important tool to gain mechanistic insight into how diet exerts metabolic and microbiome modulations, and to determine how these entities interplay. Two animal experiments are presented as part of this dissertation in which the impact of dietary lipids (grain sorghum lipid extracts and plant sterol esters) was examined in the context of dyslipidemia. GM and metabolic alterations as a result of the dietary intervention were investigated. The data revealed that the observed shifts in GM composition were most likely a response to host metabolic changes that affected the GIT environment, namely, cholesterol concentrations in the GIT were determined to impact bacterial populations in the GIT.

Although animal experiments are key to gain a mechanistic understanding of the host-microbe interactions, human trials are necessary as a direct extrapolation cannot be made from animal experiment investigations. In another set of experiments, the impact of resistant starches and whole grains was evaluated in human subjects. The studies conducted determined the impact of these dietary components on the GM in terms of dynamics, individuality of response, and their association with host

metabolic and immune markers related to metabolic disorders. Significant alterations in GM composition were observed in both studies, and an anti-inflammatory effect of whole grains was detected.

An important contribution of these studies was the use of massive parallel sequencing technology in the characterization of the GM, which permitted an overall community-wide perspective on the effects of diet on the microbiome. Moreover, dietary components that had not been previously investigated in terms of their impact on the GM were used in these studies: grain sorghum lipid extracts, plant sterols esters, and chemically-modified resistant starch.

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Chapter 2

Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects

Inés Martínez, Jaehyoung Kim, Patrick R. Duffy, Vicki L. Schlegel, and Jens Walter. *PLoS One*, 5:e15046, 2010.

2.1 Abstract

2.1.1 Background

To systematically develop dietary strategies based on resistant starch (RS) that modulate the human gut microbiome, detailed *in vivo* studies that evaluate the effects of different forms of RS on the community structure and population dynamics of the gut microbiota are necessary. The aim of the present study was to gain a community wide perspective of the effects of RS types 2 (RS2) and 4 (RS4) on the fecal microbiota in human individuals.

2.1.2 Methods and Findings

Ten human subjects consumed crackers for three weeks each containing either RS2, RS4, or native starch in a double-blind, crossover design. Multiplex sequencing of 16S rRNA tags revealed that both types of RS induced several significant compositional alterations in the fecal microbial populations, with differential effects on community structure. RS4 but not RS2 induced phylum-level changes, significantly increasing Actinobacteria and Bacteroidetes while decreasing Firmicutes. At the species level, the changes evoked by RS4 were increases in *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, while RS2 significantly raised the proportions of *Ruminococcus bromii* and *Eubacterium rectale* when compared to RS4. The population shifts caused by RS4 were numerically substantial for several taxa, leading for example, to a ten-fold increase in bifidobacteria in three of the subjects, enriching them to 18-30% of the fecal microbial community. The responses to RS and their magnitudes varied between individuals, and they were reversible and tightly associated with the consumption of RS.

2.1.3 Conclusions

Our results demonstrate that RS2 and RS4 show functional differences in their effect on human fecal microbiota composition, indicating that the chemical structure of RS determines its accessibility by groups of colonic bacteria. The findings imply that specific bacterial populations could be selectively targeted by well designed functional carbohydrates, but the inter-subject variations in the response to RS indicates that such strategies might benefit from more personalized approaches.

2.2 Introduction

The gastrointestinal microbiota is of profound importance for the human host, affecting its metabolism, immune functions, and physiology with implications to health (Gordon and Pesti, 1971, Nicholson et al., 2005). Not only are these microbial populations involved in the prevention of gastrointestinal infections and stimulation of the immune system, but recent research has indicated a role of the gut microbiome in complex diseases such as colon cancer, obesity, type 2 diabetes, and inflammatory bowel disease (Cani et al., 2007, Flint et al., 2007, Tannock, 2008). The implications of these cohesions cannot be overstated; if the gut microbiota influences health, it stands to reason that dietary factors which influence species composition and metabolic characteristics of the gut microbiota are strong candidates for disease prevention and intervention. Dietary components that are resistant to human digestion are considered the most significant source of nutrients for colonic bacteria, and they thus offer a promising tool for the modulation of the gut microbiota (Louis et al., 2007).

Resistant starches (RS) are starches or products of starch degradation that escape digestion and are not absorbed in the small intestine of healthy individuals (Asp, 1992). RS are classified into four categories according to the features that render it

undigestible. RS type 1 is physically inaccessible starch whereas RS type 2 (RS2) is native granular starch consisting of ungelatinized granules. RS type 3 is retrograded amylose, and finally, RS type 4 (RS4) is chemically modified to achieve undigestibility. Several studies have shown RS have the potential to improve health, with one of the primary benefits being maintenance of healthy blood sugar levels (Behall et al., 2006, Nilsson et al., 2008). Though resistant to digestion in the small intestine, bacterial species that reside in the colon are capable of utilizing RS as a substrate. These fermentations lead to an increase of short chain fatty acids (SCFA), especially butyrate, and a reduction of secondary bile acids, phenol, and ammonia (Louis et al., 2007, Nugent, 2005). These metabolic effects are likely to underlie some of the documented health benefits of RS, which include the prevention of colon cancer development and colitis in animal models (Bauer-Marinovic et al., 2006, Le Leu et al., 2007, Moreau et al., 2003, Toden et al., 2006).

Several studies have been performed to characterize the potential of RS to induce alterations in the composition of the gut microbiota. Increases in bifidobacteria (Conlon and Bird, 2009, Drzikova et al., 2005, Kleessen et al., 1997, Wang et al., 2002) and Bacteroides (Lesmes et al., 2008) as well as decreases in enterobacteria and *Bacteroides* (Silvi et al., 1999) have been reported. Unfortunately, most studies have been performed in either in vitro systems or animal models (Crittenden and Playne, 2006). To our knowledge, there were two previous studies that have used culture independent methods to characterize the effect of RS in humans in vivo. Abell and coworkers, who used denaturing gradient gel electrophoresis (DGGE) to study the impact of RS2 on the human gut microbiota and revealed an enrichment of phylotypes related to *Ruminococcus bromii* (Abell et al., 2008). Walker et al. (2011) detected an enrichment of bacteria related to *Eubacterium rectale* and *Ruminococcus bromii* when RS3 was consumed by overweight individuals (Walker et al., 2011).

Recently, the ecological study of the human gastrointestinal microbiota has gained enormous momentum through the development of high throughput multiplex sequencing of 16S rRNA tags (Robinson et al., 2010, Hamady and Knight, 2009). This technique has been extremely valuable, for example, in the characterization of the human microbiota in terms of lean and obese physiological states, impact of antibiotics, and the importance of delivery mode at birth (Dethlefsen et al., 2008, Dominguez-Bello et al., 2010, Turnbaugh et al., 2009). Pyrosequencing has significant advantages over other molecular techniques currently used to study microbial communities. First, unlike probe based techniques such as fluorescence in-situ hybridization (FISH), pyrosequencing allows the determination of the entire phylogenetic spectrum of the bacterial populations in one single analysis. Second, it further allows an immediate taxonomic characterization and the flexibility to analyze the communities at different taxonomic levels. Third, pyrosequencing has a markedly increased dynamic range when compared to more traditional fingerprinting techniques such as DGGE (Hamady and Knight, 2009).

The goal of the present study was to obtain a community wide perspective of the impact of RS on the composition of the human gut microbiota. We were further interested to compare RS4 and RS2 in this respect because most emphasis in the literature has been placed on the latter substrate. To achieve our goal, we conducted a placebo-controlled, double-blind crossover trial with 10 human subjects and performed a comprehensive characterization of their fecal microbiota by using a combination of approaches, including pyrosequencing of 16S rRNA tags (Figure 2.1).

Figure 2.1: **Experimental design used in this study.** Subjects (n = 10) participated in a 17-week double-blind crossover design, in which 3 dietary treatments were assessed: 100 g of crackers containing either native starch or 33 g of RS2 or RS4. An initial baseline period was proceeded by 3-week periods of each dietary treatment in succession interspersed by 2-week washout periods, and a final washout period. Weekly fecal samples were collected throughout the entire study.

2.3 Materials and methods

The human trial of this study was approved by the Institutional Review Board of the University of Nebraska (IRB Approval Number: 2008038840EP), and written informed consent has been obtained from all subjects.

2.3.1 Preparation of RS crackers

Three types of crackers, containing either RS2 (Hi-Maize 260, National Starch and Chemical Corp., Bridgewater, N.J., USA), RS4 (Fibersym RW, MGP Ingredients, Atchison, Kansas, USA), or native wheat starch (Midsol 50, MGP Ingredients, Atchison, Kansas, USA), were prepared at the American Institute of Baking International (Manhattan, Kansas). Fibersym RW is a chemically modified phosphorylated cross-linked type 4 RS prepared from wheat starch (RS4) (Woo and Seib, 2002). The crackers containing RS were formulated to both contain 33 g of fiber in the form of RS per 100 g of crackers, calculated based on the proportion of total dietary fiber (true RS) in Hi-Maize 260 (60%, dry basis) and Fibersym RW (85%, dry basis), us-

ing native wheat starch to account for the different RS contents of Hi-Maize 260 and Fibersym RW. The formulations of all three types of crackers are shown in Table 2.4, and the baking conditions of dough are presented in Table 2.5. The amount of RS in the final products was confirmed using the AOAC 991.41 method, which measures total fiber (and the most reliable method to measure RS4). This analysis revealed that the amount of fiber per 100 g of crackers after processing was 4.53 ± 1.4 g for the control crackers, 33.2 ± 4.2 g for the RS2 crackers and 30.5 ± 3.5 g for the RS4 crackers.

2.3.2 Experimental design of human study

A double-blind, crossover study was performed starting with 13 healthy human subjects. None of the subjects had been on antibiotics or on a vegetarian diet within three months prior to the start of the study or throughout its duration. Three subjects stopped their participation for reasons unrelated to the study. Thus, the study was completed by ten subjects (five males and five females) between 23 and 38 years of age. The study was conducted over a 17-week period, beginning with a two-week baseline period (no crackers administered). The subjects then consumed 100 g per day of the different crackers in sequence, each for three weeks, interspersed by 2-week washout periods. The study finished with a 2-week washout. Fecal samples were collected weekly, resulting in a total of 161 fecal samples for the entire study. For reasons unrelated to the study, we were unable to collect a total of 9 fecal samples distributed among five subjects. All missing samples corresponded to washout periods, and their omission did therefore not affect the statistical analysis.

Subjects completed a symptoms diary to assess the potential side effects of RS administration. The symptoms included were bowel movement, stool consistency, dis-

comfort, flatulence, abdominal pain, and bloating, and subjects were asked to score them on a scale from 1 (none, normal, good well-being) to 5 (severe symptoms and discomfort).

2.3.3 Collection of fecal samples and analysis by selective culture

Fresh fecal samples were processed within an hour of defecation. A ten-fold dilution of each sample in sterile phosphate buffered saline (PBS) (pH 7.0) was immediately frozen at -80°C for later DNA extraction (see below). Samples were further introduced into an anaerobic chamber (Bactron IV Anaerobic Chamber, Shel Lab, USA), and a 10-fold dilution series was made with pre-reduced sterile saline (0.9% NaCl). Aliquots were plated on Brain Heart Infusion agar (BD, USA) for the enumeration of total anaerobes (2 days), Rogosa SL (BD) for bifidobacteria (4 days), and Bacteroides Bile Esculin Agar (BD) for *Bacteroides/Parabacteroides* spp. (2 days), and the plates were incubated anaerobically at 37°C. Dilution series were plated aerobically on MacConkey agar (BD,) for enterobacteria (1 day); and Bile Esculin Azide Agar (Acumedia, USA) for enterococci (2 days); plates were incubated aerobically at 37°C. Fecal samples (2 gram) were homogenized with distilled water to obtain a slurry for pH measurements, which were performed using an Ag/AgCl pH meter (Accumet Basic AB15 pH meter, Fisher Scientific).

2.3.4 DNA extraction from fecal samples

Fecal homogenates were thawed and transferred to sterile bead beating tubes (Biospec products, Bartlesville, OK, USA) containing 300 mg of zirconium beads. Cells were recovered by centrifugation (8,000 xg for 5 min at room temperature) and suspended

in ice-cold PBS to wash the cells. This step was repeated twice before cell pellets were suspended in 100 l of lysis buffer (200 mM NaCl, 100 mM Tris, 20 mM EDTA, 20 mg/ml Lysozyme, pH 8.0) containing 20 mg/ml of Lysozyme (Sigma-Aldrich) and incubated at 37C for half an hour. Buffer ASL (1.6 ml) from QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added to each sample, after which the samples were homogenized in a MiniBeadbeater-8 (BioSpec Products, OK, USA) for 2 min at maximum speed. 1.2 ml of supernatant was used to purify DNA with the QIAamp DNA Stool Mini Kit following the manufacturer's instructions.

2.3.5 Pyrosequencing of 16S rRNA tags

The V1-V3 region of the 16S rRNA gene was amplified by PCR from fecal DNA. The 16S primers were modified to work with the Roche-454 Titanium adapter sequences and contain the A or B Titanium sequencing adapter (shown in italics), followed immediately by a unique 8-base barcode sequence (BBBBBBBB) and finally the 5 end of primer. A mixture (4:1) of the primers B-8FM (5- CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCMTGGCTCAG3) and B-8FMBifido (5-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGGGTTCGATTCTGGCTCAG3) were used as the forward primer during PCR. As the reverse primer, the primer A-518R (5- CCATCTCATCCCTGCGTGTCTCCGACTCAGBBBBBBBBBATTACCGCGGCTGCTGG 3) was used. Individual samples were amplified with primers containing unique barcodes, which allowed the mixing of PCR products from multiple samples in a single run, followed by bioinformatic assignation of the sequences to their respective samples via the barcode. Primer 8FMBifido was used in combination with primer 8FM to detect bifidobacteria, as species within this genus do not match the latter primer (Martínez et al., 2009). The PCR mixture contained 1 μl

of forward primer mix, 1 μl of reverse primer, 0.25 μl of Ex-Taq polymerase (TaKaRa Bio, USA), 1.5 μl of the sample, 6.25 μl of Ex-Taq buffer, 5 μl of deoxynucleotides and 37 μl of sterile dH2O were used for the reaction. The PCR program consisted of an initial denaturing step for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 57°C for 45 sec and extension at 72°C for 2 min, with a final step at 72°C for 10 min. The PCR products were quantified based on their staining intensity using the image acquisition software Genesnap (Syngene USA). PCR products were combined in equal amounts and gel purified using the QIAquick Gel Extraction Kit (Qiagen, USA).

Pyrosequencing was performed by the Core for Applied Genomics and Ecology (CAGE, University of Nebraska) from the A end with the 454/Roche A sequencing primer kit using a Roche Genome Sequencer GS-FLX following manufacturer's protocol for the Roche 454 GS FLX Titanium. Sequences were binned according to barcode using the Initial Process tool of the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (Cole et al., 2009) with default parameters (which included the removal of sequences containing at least one ambiguous nucleotide), except for the minimum sequence length, which was set to 300 bp. The quality approved sequences were trimmed to 495 bp before their submission to the sequence analyses (see below).

2.3.6 Sequence analyses to characterize microbial populations

Two independent approaches were used to analyze the sequences obtained with pyrosequencing. First, the Classifier tool (with a minimum bootstrap value of 80%) of the RDP was applied to obtain a taxonomic assignment of all sequences. This approach allowed a fast determination of the proportions of bacterial groups at different

taxonomic levels (phylum, family, genus). Second, sequences were assigned to Operational Taxonomic Units (OTUs) that were quantified in individual subjects. As the entire data from the ten subjects contained too many sequences for a quality alignment, sequences were aligned by subject using the Aligner web tool of the RDP, and then clustered using the Complete Linkage Clustering tool (with a maximum distance cutoff of 97%). OTUs that contained less than three sequences were excluded from the analyses. ANOVA was used to identify OTUs that were significantly affected by the dietary treatments in each of the ten subjects. These OTUs were subjected to a taxonomic classification and grouped according to phylum (Firmicutes, Bacteroidetes, and Actinobacteria). Within these phyla, five random sequences of each OTU identified above were aligned with the most closely related type strains and entry in the NCBI database using Muscle 3.6 (Edgar, 2004). Phylogenetic trees were built with MEGA 4.0 Software (Tamura et al., 2007) by neighbor-joining with 1,000 bootstrap replicates. These trees allowed us to visually assign OTUs as sequence clusters which, in most cases, encompassed sequences from several subjects, and consensus sequences were generated for each OTU. A local nucleotide database was established for each subject in BioEdit(Hall, 1999) containing all sequences detected by pyrosequencing, and the BLASTn algorithm was used with a 97% cutoff (minmum length 300 bp) to quantify each OTU in the fecal bacterial populations in each sample. We verified that this approach did not result individual sequences being assigned to different OTUs. In two occasions, two OTUs that were initially identified as distinct had very high sequence similarities, and were thus combined.

Diversity of the fecal microbiota was determined using 16S rRNA sequence data with two different methods, Shannon's index and the generation of rarefraction curves. The DNA sequences of each sample were individually aligned and clustered using Aligner and Complete Linkage Cluster tools of the RDP. Individual cluster files cor-

responding to each fecal sample were used to determine the Shannon's Index and construct Rarefraction curves.

2.3.7 Analysis of fecal microbiota by PCR-DGGE

PCR-DGGE and quantitative analysis of molecular fingerprints was performed as described previously (Martínez et al., 2009). Briefly, PCR was performed using primers PRBA338fGC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA-CGGGGGGACTCCTACGGGAGGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGCTGC-TGG-3') (Ovreas et al., 1997). DGGE was done as described by Walter and co-workers (2001) using a DCode universal mutation detection system (Bio-Rad, Hercules, USA), and DGGE profiles were analyzed using BioNumerics software Version 5.0 (Applied Maths). Band staining intensities were calculated as percent peak area in relation to the total peak area of the entire fingerprint. DNA fragments whose staining intensity changed according to dietary treatment were excised, purified as described by Walter and coworkers (Walter et al., 2001), and cloned using the TOPO TA Cloning Kit for Sequencing (pCR 4 TOPO Vector) (Invitrogen). Plasmids were isolated from transformants using the QIAprep Spin Minprep kit (Qiagen, Hilden, Germany), and inserts were sequenced by a commercial provider. Closest relatives of the partial 16S rRNA sequences were determined using the SeqMatch web tool provided through the Ribosomal Database Project.

2.3.8 *Bifidobacterium* specific qRT-PCR

Quantitative real time PCR (qRT-PCR) was performed as described by Martínez et al. (2009), using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany) and the *Bifidobacterium*-specific primers (F: 5TCGCGTC(C/T)GGTGTGAAAG3)

and R: 5CCACATCCAGC(A/G)TCCAC3) (Rinttila et al., 2004). Standard curves for absolute quantification of bifidobacteria in the fecal samples were prepared using overnight cultures (14 h) of *Bifidobacterium animalis* ATCC 25527^{T} and *Bifidobacterium infantis* ATCC 15697^{T}.

2.3.9 Statistical analysis

One-way ANOVA tests with repeats were performed to identify differences in fecal microbiota composition induced through the dietary treatments (RS2, RS4 and control) in all ten subjects. One-way ANOVA tests were performed to identify significant alterations of taxa in individual subjects. Samples obtained during the baseline and washout periods were not included into the statistical analysis. Post hoc pair-wise comparisons were done using Tukey's method. P-values <0.05 were considered significant unless otherwise stated.

2.4 Results

2.4.1 Multiplex sequencing of 16S rRNA tags revealed alterations of the fecal microbiota through RS consumption and functional differences between RS types 2 and 4

Pyrosequencing of 16S rRNA amplicons from 161 fecal samples resulted in an average of 3,423 sequences per sample after quality control (551,183 sequences in total) with a mean sequence length of approximately 490 bp. The average number of operational taxonomic units (OTUs) identified per subject was 1,081. Rarefaction curves for all

ten subjects and the three treatments and baselines/washouts were generated and are shown (Supplementary Figure 2.5). This analysis and diversity examination by Shannon's index revealed that the consumption of RS did not alter the bacterial diversity in fecal samples (Supplementary Figure 2.5).

The bacterial composition in the ten subjects during the baseline period was, as shown by other studies (Turnbaugh et al., 2009, Ley et al., 2006b), dominated by the phyla Firmicutes (78%) and Bacteroidetes (13%). Other phyla detected were Actinobacteria (3%), Verrucomicrobia (1%), and Proteobacteria (1%), and 4% of the sequences remained unclassified (Supplementary Figure 2.6A). At the family level, the predominant groups were the Lachnospiraceae (42%), Ruminococcaceae (19%), Bacteroidaceae (8%) (Supplementary Figure 2.6B). Among the well characterized culturable genera were *Bacteroides* (7.5%), *Bifidobacterium* (1.3%), *Fecalibacterium* (8.4%), *Ruminococcus* (2.5%), *Roseburia* (2.1%), and *Dorea* (3.2%) (Supplementary Figure 2.6C).

Sequence proportions determined by pyroseqeuncing were used to establish the effects of RS on the gut microbiota composition, and the groups of colonic bacteria that were significantly affected are shown in Table 2.1. The control crackers included in the study (providing a daily dose of more than 55 g of native starch) did not have a significant impact on the fecal microbiota, as the microbial populations during administration of these crackers showed little difference to those during baseline and washout periods. In contrast, RS significantly affected several groups of colonic bacteria, with the two types of RS exerting functional differences in terms of their ability to modulate the gut microbiota. Taxonomy-based analysis using RDP Classifier revealed major differences in the proportions of phyla associated with consumption of RS4, including significant decreases in Firmicutes ($p<0.001$) by more than 10% on average, and increases in Bacteroidetes ($p<0.01$) and Actinobacteria ($p<0.05$) by

Table 2.1: **Abundance of bacterial taxa that were impacted by RS consumption in fecal samples of ten human subjects as determined by pyrosequencing of 16S rRNA tags.** Proportion of bacterial taxa expressed in percentage (Mean ± SD)

	RS2[1]	RS4[1]	Control [1]	Baseline[2]	Washout[3]	P-value[4]
Phylum						
Firmicutes	**75.9 ± 13.4**	*65.6 ± 15.0*	**79.6 ± 9.6**	78.2 ± 7.5	78.1 ± 8.5	0.0010
Bacteroidetes	*10.1 ± 6.6*	**16.3 ± 9.7**	*10.4 ± 6.9*	12.7 ± 6.5	12.2 ± 5.8	0.0028
Actinobacteria	6.1 ± 6.4	**11.4 ± 12.5**	*4.1 ± 3.1*	3.1 ± 2.5	4.1 ± 3.2	0.0334
Family						
Bifidobacteriaceae	5.8 ± 6.0	**11.1 ± 11.7**	*3.0 ± 2.5*	2.1 ± 1.7	2.8 ± 2.2	0.0262
Porphyromonadaceae	*0.6 ± 1.0*	**3.4 ± 1.9**	*0.5 ± 0.3*	0.6 ± 0.4	0.5 ± 0.4	0.0002
Ruminococcaceae	**24.8 ± 13.6**	*16.7 ± 7.4*	**23.2 ± 9.7**	19.3 ± 7.4	20.7 ± 7.6	0.0031
Erysipelotrichaceae	3.1 ± 2.8	*2.6 ± 2.6*	**3.9 ± 3.2**	4.7 ± 4.9	3.9 ± 3.1	0.0279
Genus						
Faecalibacterium	9.7 ± 4.4	*7.8 ± 3.4*	**10.8 ± 4.7**	8.4 ± 4.2	8.8 ± 2.9	0.0336
Parabacteroides	*0.6 ± 1.0*	**3.4 ± 1.9**	*0.4 ± 0.5*	0.5 ± 0.3	0.5 ± 0.4	0.0002
Bifidobacterium	4.5 ± 4.9	**8.9 ± 10.2**	*2.2 ± 1.7*	1.5 ± 1.3	2.1 ± 1.6	0.0342
Dorea	*1.7 ± 1.2*	*1.6 ± 1.2*	**3.0 ± 2.0**	2.9 ± 2.2	2.7 ± 2.0	0.0030
Species (OTU)						
B. adolescentis	3.7 ± 4.5	**7.9 ± 10.3**	*1.7 ± 1.9*	1.5 ± 1.2	1.8 ± 1.3	0.0347
P. distasonis	*0.2 ± 0.4*	**1.5 ± 1.0**	*0.2 ± 0.1*	0.2 ± 0.1	0.2 ± 0.2	0.0002
R. bromii	**4.1 ± 5.1**	*1.2 ± 1.3*	2.6 ± 3.2	1.0 ± 1.1	2.0 ± 1.5	0.0479
F. prausnitzii	4.8 ± 2.6	*3.6 ± 2.0*	**5.6 ± 3.1**	4.2 ± 2.8	4.2 ± 2.4	0.0301
E. rectale	**8.3 ± 7.1**	*3.4 ± 2.3*	4.9 ± 4.0	5.4 ± 2.9	4.7 ± 2.0	0.0301
D. formicigenerans	1.2 ± 1.0	*1.0 ± 1.1*	**2.2 ± 1.6**	2.3 ± 1.8	1.9 ± 1.7	0.0140
C. clostridioforme	2.6 ± 2.4	**3.4 ± 2.5**	*1.2 ± 0.8*	1.4 ± 1.3	1.5 ± 1.2	0.0126
Clostridiales spp.	*0.3 ± 0.6*	**0.9 ± 0.9**	0.7 ± 0.8	0.2 ± 0.4	0.8 ± 0.7	0.0322

[1] The bacteria populations are averages of all three time points of feeding periods.
[2] The bacteria populations are averages of the two time points of the baseline period.
[3] The bacteria populations are averages of all the six time points of the three washout periods.
[4] Bacterial populations during the dietary treatments were compared to each other with repeated measures ANOVA and Tukey's post hoc test. Numbers in bold represent proportions that were significantly higher than numbers shown in italic.

around 5% each 2.6. These changes were associated with a decrease in the family Ruminococcaceae ($p<0.01$) and increases in the genera *Parabacteroides* ($p<0.001$) and *Bifidobacterium* ($p<0.05$). The proportion of the genus *Faecalibacterium* decreased in the RS4 treatment ($p<0.05$), although this reduction was small (less than 1% when compared to the baseline) (Table 2.1). The genus Dorea was determined to be significantly reduced by both types of RS ($p<0.01$).

To gain a more in depth understanding of the effects of RS on the relative abundances of microbial taxa, we used a phylogeny-based strategy to analyze the sequence data on the basis of OTUs (>97% sequence identity).

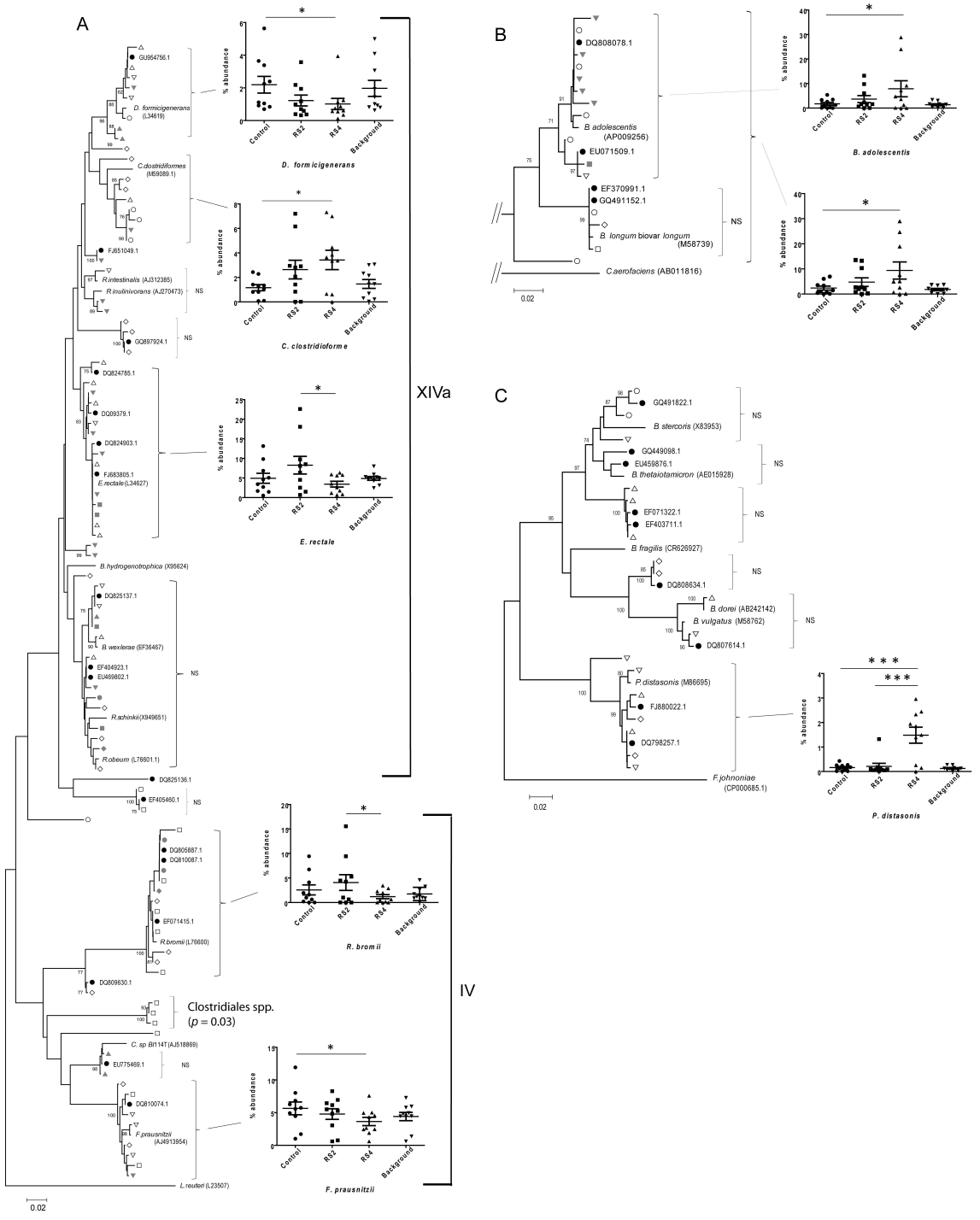

Figure 2.2: **Characterization of the fecal microbiota in ten human subjects that consumed a random succession of crackers containing RS2, RS4, and native wheat starch (control) by multiplex pyrosequencing of 16S rRNA tags.** Phylogenetic trees that encom-

pass the phyla (A) Firmicutes (with Clostridiales groups XIVa and IV labeled), (B) Actinobacteria and (C) Bacteroidetes are shown. The trees contain representative sequences of all OTUs detected to be impacted by RS in individual subjects together with sequences of related entries in the database (which included both type strains of known species and sequences from molecular studies of human fecal samples). Sequences were aligned in Muscle 3.6 and the trees were built using the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA 4.0. Open-black and closed-gray symbols were used to label sequences from individual subjects. OTUs that were not significantly affected in all ten subjects were labeled as not significant (NS). The graphs next to the trees show the abundance of OTUs and bacterial groups that were significantly altered in the treatment groups (RS2, RS4, control). These graphs show mean proportions of the three individual samples taken during the treatment periods for each subject. Background refers to samples taken in periods were no crackers were consumed. Repeated measures ANOVA in combination with a Tukey's post-hoc test were performed to indentify differences between treatment groups, and the background was not included in the statistic analysis. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

First we identified OTUs that were affected through the dietary treatments in individual subjects. We then constructed phylogenetic trees with representative sequences of these OTUs according to phylum, which are shown in Figure 2.2A (for Firmicutes), Figure 2.2B (for Actionobacteria), and Figure 2.2C (for Bacteroidetes). The abundance of the OTUs in all ten subjects was then quantified by local BLASTn. This analysis revealed that eight OTUs showed statistically significant differences among treatment groups, seven of which could be linked to known bacterial species (Table 2.1, Figure 2.2). The findings of the OTU-based approach were in general agreement with those obtained with the Classifier tool, and the species responsible for the significant RS induced changes in the genera *Bifidobacterium*, *Parabacteroides*, *Faecalibacterium*, and *Dorea* did correspond to *Bifidobacterium adolescentis* ($p<0.05$), *Parabacteroides distasonis* ($p<0.001$), *Faecalibacterium prausnitzii* ($p<0.05$) and *Dorea formicigenerans* ($p<0.05$), respectively (Table 2.1). In addition, the OTU-based analysis iden-

tified four additional taxa that differed between the treatment groups, belonging to the Clostridium clusters XIVa and IV. The proportion of *Clostridium clostridioforme* was increased by both RS, and the increase reached statistical significance for RS4 ($p<0.05$). Furthermore, the abundance of the species *Eubacterium rectale* ($p<0.05$) and *Rumminococcus bromii* ($p<0.05$) were significantly increased when RS2 was consumed when compared to RS4.

2.4.2 The population shifts induced by RS were substantial but varied between subjects

RS, and especially RS4, led to major changes in the composition of the gut microbiota in a subset of subjects. Numerically, the most substantial alterations were the change in the genus *Bifidobacterium* (e.g. *Bifidobacterium adolescentis*), which increased approximately 10 fold (from 2-3% to 18-30%) in three subjects through RS4. Other significant changes were *Parabacteroides distasonis*, which significantly increased through RS4 by 7 fold on average, and *Eubacterium rectale*, which was significantly enriched when RS2 was consumed, reaching around 20% of the total population in two of the subjects. The most consistent alteration detected was the reduction in Firmicutes by RS4, which occurred in nine of the subjects (Figure 2.3). Other common alterations were the increase in Bacteroidetes (seven subjects), *Parabacteroides distasonis* (seven subjects), and *Bifidobacterium adolescentis* (six subjects) through RS4, and the increase of *Eubacterium rectale* through RS2 (eight subjects). Despite these substantial population shifts, our findings clearly showed that effects of RS and their magnitude varied among individuals. Figure 2.3 shows compositional changes induced by RS2 and RS4 when compared to administration of native starch for individual subjects. The data revealed that none of the community shifts induced through

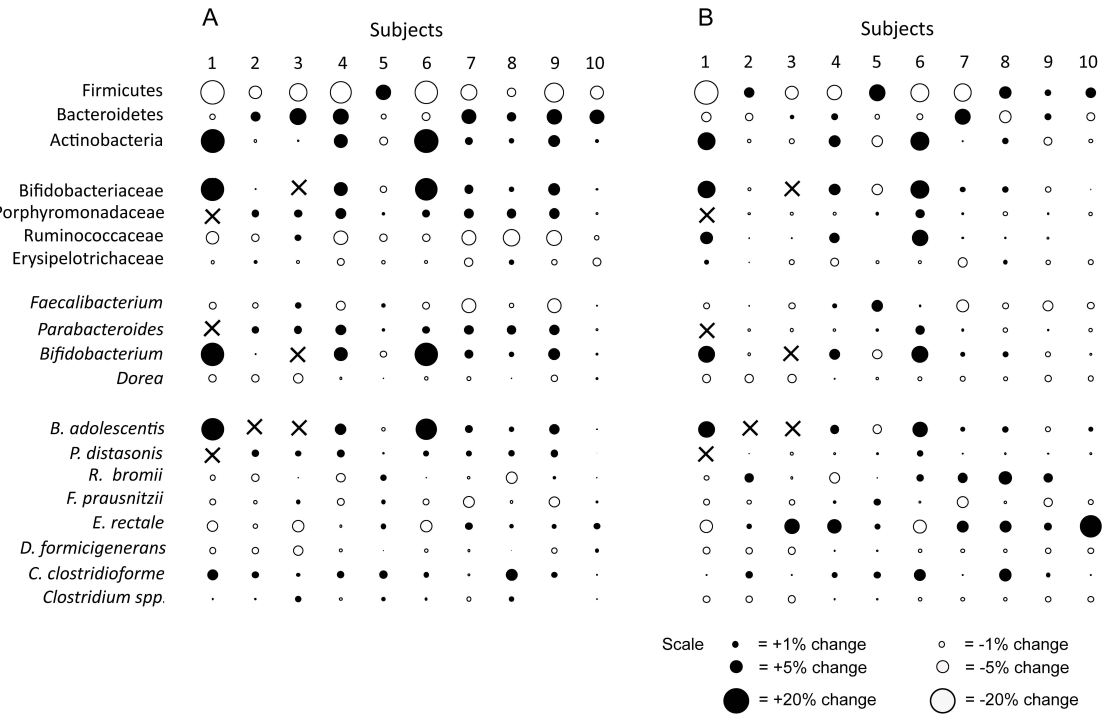

Figure 2.3: **Bubble plots showing differences in the proportions of bacterial taxa (as per cent of the total microbiota composition) detected between the RS4 (A) and RS2 (B) periods when compared to the control period.** The sizes of the bubbles are proportional to the magnitude of the difference. Black circles represent increases in proportions induced through RS treatment, and white circles show a decrease.

the two RS types were observed in all ten subjects.

2.4.3 Temporal dynamics of microbial populations in response to RS

The generation of community profiles from 17 individual samples per subject throughout the trial allowed insight into how RS influenced the population dynamics within the fecal microbiota. This analysis showed that all the changes induced by RS were reversible within one week, and no differences in the proportions of the bacterial

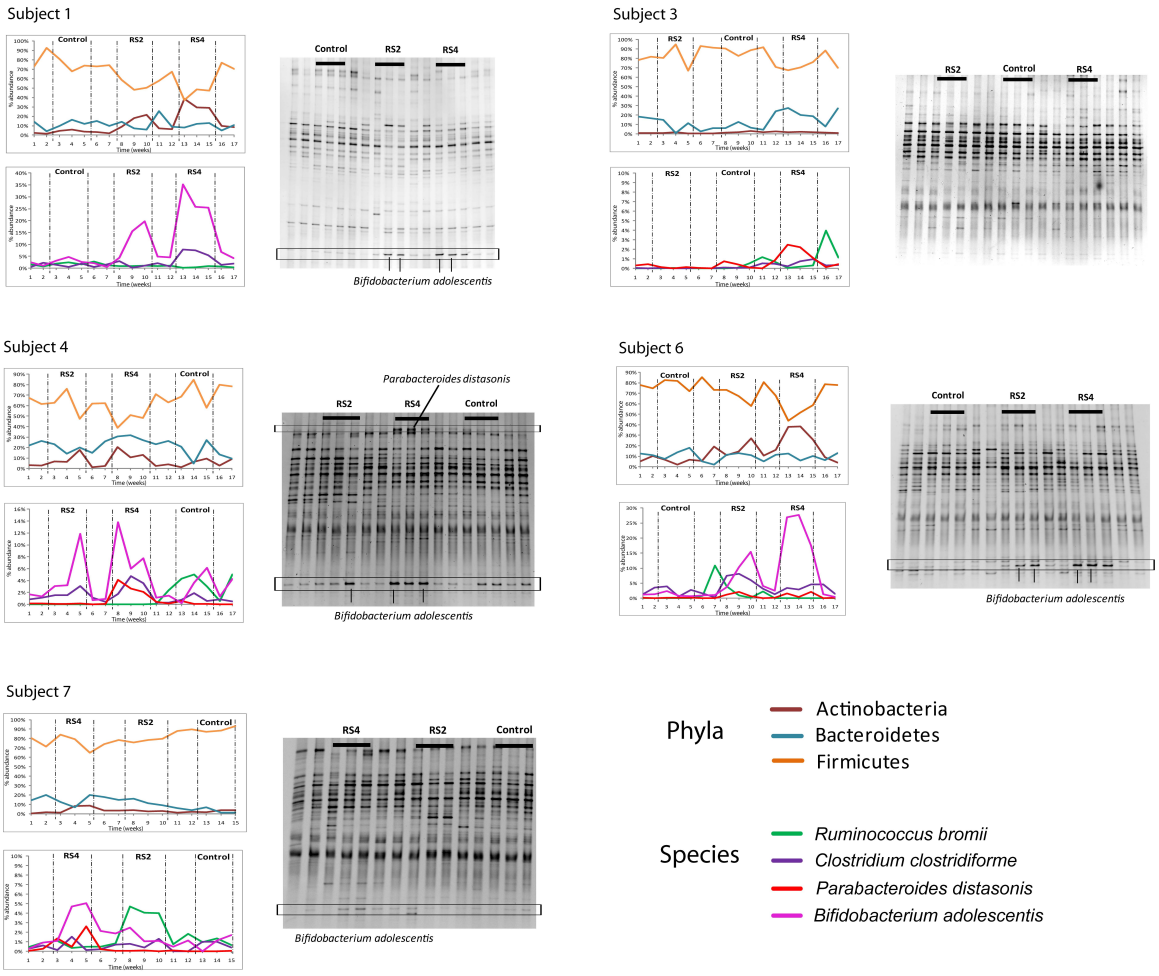

Figure 2.4: **Temporal dynamics of the human fecal microbiota in response to the consumption of crackers containing RS2, RS4, and native wheat starch (control) in five human subjects.** Graphs on the left show proportions of the three main phyla and four representative species (*Bifidobacterium adolescentis*, *Parabacteroides distasonis*, *Ruminococcus bromii* and *Clostridium clostridioforme*) as determined by pyrosequencing of 16S rRNA tags. Gel images on the right show molecular fingerprints generated by DGGE. Bands that represent *Bifidobacterium adolescentis* and *Parabacteroides distasonis* are labeled.

groups were detected between the first washout sample and the baseline (Student's t-test, $p>0.05$). Figure 2.4 shows the temporal patterns of the three main phyla (Actinobacteria, Bacteroidetes, and Firmicutes) and four selected species (*Ruminococcus bromii*, *Clostridium clostridioforme*, *Parabacteroides distasonis* and *Bifidobacterium adolescentis*) for five representative subjects. The data revealed that bacterial groups showed marked differences in the stability of their populations and in their temporal response to RS. For example, levels of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* were remarkably stable in fecal samples in baseline and washout samples, and their populations returned to baseline level within one week after RS administration was stopped. In contrast, proportions of some taxa, e.g. the species *Ruminococcus bromii* and *Clostridium clostridioforme* showed higher fluctuations in background samples (Figure 2.4). Although all these taxa were also significantly impacted by dietary RS, population dynamics were more idiosyncratic, and these bacterial groups might be more influenced by other dietary components or environmental factors.

The analysis of population dynamics revealed that RS2 and RS4 induced changes within the fecal microbial community that differed in their temporal patterns. RS4 led to an abrupt increase in the abundance of *Bifidobacterium adolescentis* in some subjects with a slight mitigation throughout the three-week feeding period (subject 1, 4, and 6). Out of the six subjects that showed an increase in *Bifidobacterium adolescentis* with RS4, five did also manifest an increase with RS2. However, RS2 induced a slower gradual increase with higher proportions in week three of consumption when compared to week one.

2.4.4 Selective culture, PCR-DGGE and Bifidobacterium specific quantitative RT-PCR (qRT-PCR)

Quantification of bacterial taxa in human fecal samples by pyrosequencing of 16S rRNA tags has been validated and it showed a high correlation with other molecular methods such as qRT-PCR and phylogenetic microarray (Claesson et al., 2009, Martínez et al., 2009). To investigate the impact of RS on human fecal microbiota with independent and well established methods, we analyzed all 161 fecal samples obtained during this study with selective culture for representative bacterial groups, PCR-DGGE, and *Bifidobacterium* specific qRT-PCR.

Throughout the study, numbers of total anaerobic bacteria, enterobacteria, enterococci, bifidobacteria, and *Bacteroides/Parabacteroides* spp. were determined by selective culture. These bacterial taxa were selected as they were included in previous studies that concerned the effect pre- and probiotics on the human fecal microbiota, and enterococci and enterobacteria cannot be detected by pyrosequencing as they constitute a minor fraction of the total microbial community in the human gut (Tannock et al., 2004, Tannock GW, 2000). Selective culture revealed significant higher numbers of bifidobacteria and *Bacteroides/Parabacteroides* spp. during RS4 consumption ($p<0.05$) (Table 2.2), confirming the findings obtained by pyrosequencing (Table 2.1). No significant changes were detected for enterococci and lactose fermenting enterobacteria (Table 2.2).

DGGE revealed that RS4, and to a lesser degree RS2, induced significant alterations to the fecal microbiota of most subjects. The most consistent change detected was a significant increase in the staining intensity of a DNA fragment that represented *Bifidobacterium adolescentis*, which occurred in four subjects during RS4 consumption and in 3 subjects when RS2 was consumed. The intensity of this DGGE band

in all the subjects showed a remarkably high correlation ($r = 0.9178$, $p<0.0001$) with the abundance of *Bifidobacterium adolescentis* assessed by pyrosequencing (Supplementary Figure 2.7A). DGGE analysis confirmed the distinct dynamics of the *Bifidobacterium adolescentis* population in response to RS2 and RS4 that were detected by pyrosequencing, meaning that RS4 induced a swift and reversible increase in band intensity while RS2 caused a gradual raise (Figure 2.4). DGGE also detected an increase of *Parabacteroides distasonis* in one of the subjects when RS4 was consumed. Therefore, DGGE confirmed the increase in bifidobacteria and *Parabacteroides distasonis* in some of the subjects, but as expected, overall resolution of this technique was lower than pyrosequencing.

Quantitative enumeration of bifidobacteria by qRT-PCR confirmed the significant increase in *Bifidobacterium* numbers during consumption of RS4 (10.3 ± 1.5 $\log_{10}$ cells/gram of feces; $p<0.01$), and also indicated a significant increase when RS2 (10.1 ± 1.3 $\log_{10}$ cells/gram of feces; $p<0.05$) was consumed compared to control (9.7 ± 1.2 $\log_{10}$ cells/gram of feces). The total cell numbers of bifidobacteria increased by more than three-fold on average through RS4, while RS2 doubled the numbers. In the three subjects with the highest response to RS4, qRT-PCR showed an increase of bifidobacteria to more than 10^{11} cells per gram. The cell counts obtained with qRT-PCR in all fecal samples included in this study ($n = 161$) showed a high correlation ($r=0.8310$, $p<0.0001$) with the abundance of bifidobacteria determined by pyrosequencing (Figure 2.7B).

2.4.5 RS was well tolerated by human subjects

In addition to microbiota analyses, we also collected data on bowel-related characteristics during the feeding and washout periods using a weekly symptoms diary that

rated bowel movement, stool consistency, discomfort, flatulence, abdominal pain, and bloating on a scale from 1 (best) to 5 (worse). One-way ANOVA analysis revealed a significant difference for flatulence ($p<0.05$), which was moderately increased during the consumption of both types of RS when compared to periods with control crackers (Table 2.3). No significant changes occurred in fecal pH for any of the treatments and no significant detrimental effects were observed on bowel movement, stool consistency, or discomfort, indicating that RS at doses of 33 g per day are well tolerated in human subjects.

2.5 Discussion

To gain a deeper understanding of the impact of two chemically different forms of RS on the composition and temporal dynamics of the fecal microbiota, we employed a study in 10 human subjects who were weekly monitored throughout a period of 17 weeks. The data revealed that RS types induced substrate specific shifts in the fecal microbial community that were tightly associated with consumption and which varied between subjects. Our in vivo findings on RS2 were in accordance to previous studies on starch fermentation in *in vitro* models of the large intestine, which showed an enrichment of *Bifidobacterium adolescentis*, *Eubacterium rectale*, and *Ruminococcus bromii* (Kovatcheva-Datchary et al., 2009, Leitch et al., 2007). The same bacterial groups, with the exception of bifidobacteria, were also enriched in fecal samples of obese human subjects during consumption of RS3 (Walker et al., 2011). In contrast, our findings clearly showed that the response of the fecal microbiota to RS4 differed to that of RS2 and RS3. For example, *Parabacteroides distasonis* was enriched through RS4, while *Eubacterium rectale* and *Ruminococcus bromii* showed a significant decrease. Strikingly, RS4 also led to phylum level alterations, decreasing the proportion

of Firmicutes while increasing Bacteroidetes and Actinobacteria. Such phylum level changes have not been observed in fecal samples of human subjects consuming RS2 in our study and RS3 as shown by Walker and coworkers (Walker et al., 2011).

There was very little overlap in the bacterial groups that responded to both RS2 and RS4. This was surprising as the RS types used in this study were both starch polysaccharides that consist of glucose monomers with the same covalent bonds, although the RS4 was cross-linked by phosphorylation. One of the bacterial groups that responded to both RS types was the genus *Bifidobacterium*, which increased in six subjects with RS4 and in five of the same subjects with RS2. However, the temporal dynamics of these modulations differed. RS2 led to a much slower raise in bifidobacteria, reaching comparable numbers to RS4 only in week three. This clearly showed that time is an important variable when studying dietary modulations of the human gut microbiota. It appears that the ability to increase levels of bifidobacteria is comparable between RS2 and RS4 in the long run, but extended feeding studies will be necessary to determine the exact taxon-time patterns of responses to different forms of RS.

Questions remain about the mechanisms by which different RS types selectively promote groups of colonic bacteria in humans in vivo. Starch fermentation per se should not be selective as many bacterial genera (*Clostridium*, *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Prevotella*, *Roseburia*, *Eubacterium*, *Ruminococcus*, etc.) present in the human GIT can utilize this substrate in vitro, and various bacterial systems involved in the degradation of starch have been identified by genomic approaches (Louis et al., 2007, Kovatcheva-Datchary et al., 2009, Bird and Topping, 2008, Cho et al., 2001, D'Elia and Salyers, 1996, Duncan et al., 2007, Flint et al., 2008, Ryan, 2004, Schell et al., 2002, Xu et al., 2003). However, our in vivo findings showed that substrate preferences and competitive abilities exist in the gut environment. In this respect, it is

important to point out that the RS types used in this study varied markedly in terms of their chemical structure. RS2 is a granular form of high amylose corn starch, while RS4 is a chemically modified starch that is cross-linked through phosphate moieties. Therefore, it is possible that different groups of colonic bacteria produce enzymes with distinct activities towards the two RS types, promoting different dynamics in the gut ecosystem. However, it is also of note that the ability of bacterial groups to make use of RS in vivo might relate not only to the utilization but also to the binding of the substrate. It is striking that *Ruminococcus bromii*, *Bifidobacterium adolescentis*, and *Eubacterium rectale*, which showed the most substantial increases in the human gut in response to RS2, have also been shown to form highly selective associations with this substrate (Leitch et al., 2007). Therefore, the adherence of bacteria to starch granules might constitute an important first step in the utilization of this substrate, and groups of colonic bacteria might differ in their ability to adhere to granules of RS2 and RS4. The mechanisms by which different types of RS become fermented in the human colon remain an important area of future research.

A significant finding of this study was the individualized responses of the gut microbiota to RS2 and RS4, which has also been shown previously for RS3 (Walker et al., 2011). Out of the nineteen OTUs that were detected to respond during this study in individual subjects, eleven did not reach significance when all subjects were included in the analysis (e.g. *Bifidobacterium longum*, *Ruminococcus obeum*, *Roseburia intestinalis*, *Roseburia inulinivorans*, and several *Bacteroides* spp.). In addition, none of the taxa that were significantly affected by RS showed a response in all ten subjects. There are three possible explanations for the individuality of the responses. First, few OTU are completely conserved among humans (Turnbaugh et al., 2009, Tap et al., 2009), thus species that were affected in some subjects might simply not be present in other individuals. Second, strain-level differences in the ability to utilize substrates

could contribute to the inter-individual variations. For example, it has been shown for *Bifidobacterium adolescentis* that individual strains can have major differences in their amylolytic activity (Crittenden et al., 2001, Ramsay et al., 2006). Lastly, host factors might play an important role. For example, subject specific environmental constrains (e.g. through limitations in growth factors other than carbohydrates) might restrict the ability of a bacterial group to increase in numbers even if a suitable growth substrate is provided. In addition, differences in host genotype might influence transit times or the amount of digestive enzymes secreted, thus affecting the fraction of RS that survives digestion.

There is currently no scientific consensus of what defines a healthy human microbiota in terms of its composition. Therefore, predictions on the consequences of the compositional alterations induced through RS2 and RS4 in terms of health remain speculative. Nevertheless, the distinct effects of RS2 and RS4 on the microbial community in the gut suggest that these substrates could have a different impact on the host. RS2 promoted *Eubacterium rectale*, a species associated with high butyrate production (Flint et al., 2007), a trait that could be especially beneficial in the prevention of inflammation and colon cancer (McIntyre et al., 1993, Scheppach et al., 1992). In contrast, RS4 reduced the amount of Firmicutes in favor of Bacteroidetes and bifidobacteria. Such a shift in the gut microbiota could be especially beneficial in the prevention or treatment of obesity and related metabolic disorders. A microbiome enriched in Firmicutes has been associated with an increased capacity for energy harvest and obesity (Ley et al., 2006a, Turnbaugh et al., 2006), and a reduction of this phylum could therefore reduce the amount of calories extracted from the diet. Furthermore, bifidobacteria have been linked to metabolic and immunological improvements related to type 2 diabetes (Cani and Delzenne, 2009). As we gain a better understanding about the contributions of members of the gut microbiota to

disease, knowledge as obtained during this study can aid in a more systematic selection of carbohydrates for intervention studies.

In this study, we demonstrated that RS2 and RS4 promote distinct compositional alterations within the human gut microbiota. These functional differences imply that specific bacterial populations can be selectively targeted by starches with different chemical structures. If future research will reveal causative associations between dysbiosis and disease, then selective dietary strategies that redress these imbalances have potential to improve health. However, the individualized responses observed during this study certainly pose a hurdle to developing universal dietary recommendations, and they imply that more personalized strategies that target the gut microbiome might enhance the success rate of such applications.

2.6 Supplementary material

Table 2.2: **Enumeration of bacterial groups through culturing.** Log_{10} cfu/g feces (Mean ± SD)[a].

Bacterial group	RS2	RS4	Control	Background[b]	P-value
Lactose fermenting bacteria	6.55 ± 0.83	5.87 ± 1.07	6.77 ± 0.76	6.25 ± 0.51	0.0849
Enterococci	4.05 ± 1.30	3.92 ± 1.22	4.64 ± 0.95	3.96 ± 0.41	0.2747
Bifidobacteria	9.61 ± 0.50	**9.79 ± 0.51**	*9.49 ± 0.42*	9.57 ± 0.19	0.0193
Bacteroidetes	*9.35 ± 0.41*	**9.73 ± 0.43**	*9.38 ± 0.25*	9.30 ± 0.33	0.0117
Total anaerobes	10.51 ± 0.27	10.31 ± 0.33	10.45 ± 0.12	10.41 ± 0.14	0.2791

[a] Numbers in bold represent the significantly higher values than the ones in italic.
[b] Background refers to bacterial numbers obtained from the combined baseline and wash-out periods.

Table 2.3: **Symptoms.** Mean ± standard deviations of weekly symptoms reported by the subjects in a scale from 1 (best) to 5 (worse).

	RS2	RS4	Control	Background[a]
Bowel movement	1.73 ± 0.83	1.90 ± 0.93	1.73 ± 0.77	1.74 ± 0.59
Stool consistency	2.07 ± 1.29	2.23 ± 0.92	2.03 ± 0.91	2.00 ± 0.83
Discomfort	1.65 ± 0.65	1.87 ± 0.79	1.50 ± 0.55	1.53 ± 0.40
Flatulence[b]	2.42 ± 1.28	2.27 ± 1.00	1.37 ± 0.58	1.54 ± 0.43
Abdominal pain	1.63 ± 0.79	1.47 ± 0.67	1.40 ± 0.60	1.36 ± 0.62
Bloating	1.67 ± 0.98	1.40 ± 0.52	1.07 ± 0.14	1.29 ± 0.45

[a] Background refers to bacterial numbers obtained from the combined baseline and wash-out periods.

[b] Significant differences were detected by ANOVA ($p<0.05$). Tukeys post-hoc test did not detect significance in pair wise comparisons.

Table 2.4: **Formulation of crackers per 100 g**.

Ingredient	Control	RS2	RS4
Pastry flour	1.97	1.97	1.97
Midsol 50 (native starch)	55.72	-	16.39
Hi-Maize 260	-	55.72	-
Fibersym ®RW	-	-	39.33
Soybean and cottonseed shortening	7.87	7.87	7.87
Wheat gluten	7.87	7.87	7.87
White granulated sugar	5.24	5.24	5.24
Malt extract	0.33	0.33	0.33
Sweet whey-dried	0.98	0.98	0.98
Table salt	0.66	0.66	0.66
Baking soda	0.33	0.33	0.33
Water	18.36	18.36	18.36
Ammonium bicarbonate	0.66	0.66	0.66

Table 2.5: **Baking conditions (°F) of crackers containing control starch, RS2, RS4.**

Control Doughs Oven Profile:			
	Zone 1	Zone 2	Zone 3
Top	460	470	420
Bottom	430	400	380
Bake time	7 minutes		
RS4 Doughs Oven Profile:			
	Zone 1	Zone 2	Zone 3
Top	470	450	440
Bottom	470	470	440
Bake time	7 minutes		
RS2 Doughs Oven Profile:			
	Zone 1	Zone 2	Zone 3
Top	470	450	440
Bottom	470	470	440
Bake time	8 minutes		

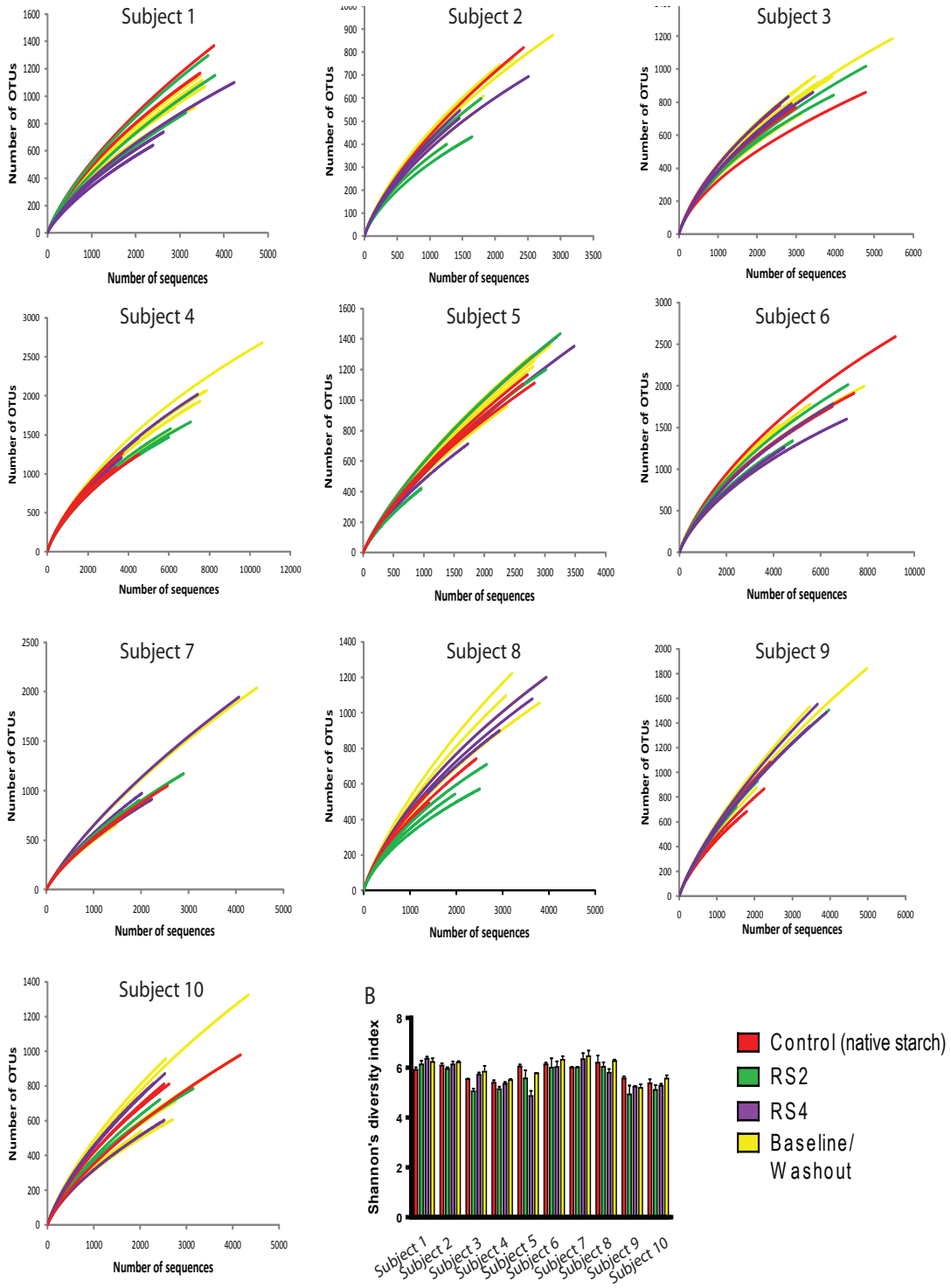

Figure 2.5: **Diversity and species richness of the fecal microbiota in ten human subjects that consumed crackers containing native starch, RS2, RS4, or no crackers**. (A) Rarefaction curves showing the amount of OTUs in all individual fecal samples taken from the ten subjects. (B) Shannon's Diversity Index for all subjects during treatments and baseline/washout. Native starch (red), RS2 (green), RS4 (purple), no crackers (yellow).

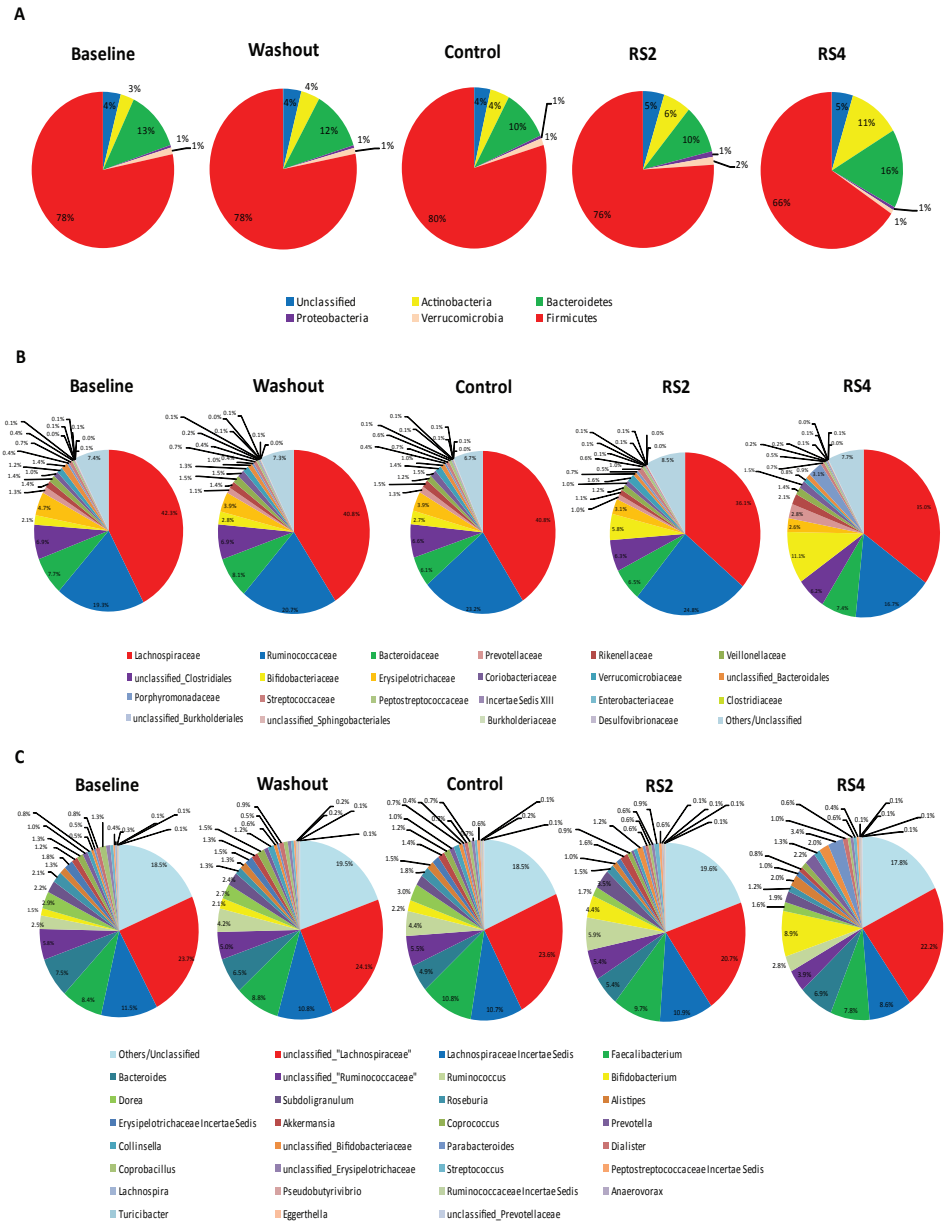

Figure 2.6: **Collective fecal microbial composition including the major taxonomic groups.** At the (A) phylum, (B) family, and (C) genus levels averaged for 10 human subjects corresponding to the baseline, washouts, and periods in which crackers containing native starch (control), RS2 and RS4 were consumed.

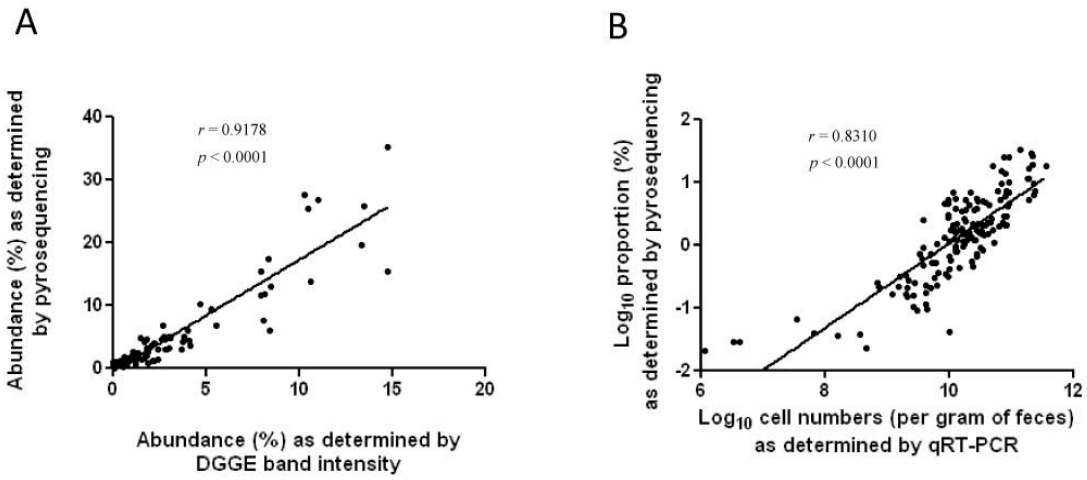

Figure 2.7: **Confirmation of findings obtained with pyrosequencing by analyzing the fecal microbiota with PCR-DGGE and *Bifidobacterium* specific qRT-PCR.** (A) Pearson correlation between the abundance of *Bifidobacterium adolescentis* as determined by band intensity in PCR-DGGE and pyrosequencing of 16S rRNA tags. (B) Pearson correlation between cell numbers and percent abundance of bifidobacteria as determined by qRT-PCR and pyrosequencing, respectively.

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Chapter 3

Whole grains induce immunological improvements in humans that are linked to the composition of the gut microbiome

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3.1 Abstract

The involvement of the gut microbiota in metabolic disorders, and the ability of whole grains to affect both host metabolism and gut microbial ecology, suggest that some benefits of whole grains are mediated through their effects on the gut microbiome. Nutritional studies that assess the effect of whole grains on both the gut microbiome and human physiology are needed. We conducted a randomized cross-over trial in which 28 healthy humans consumed a daily dose of 60 grams of whole grain barley (WGB), brown rice (BR), or an equal mixture of the two (BR+WGB), and characterized their impact on fecal microbial ecology and blood markers of inflammation, glucose and lipid metabolism. All treatments increased microbial diversity, the Firmicutes/Bacteroidetes ratio, and the abundance of the genus *Blautia* in fecal samples. The inclusion of WGB enriched the genera *Roseburia*, *Bifidobacterium*, and *Dialister*, and the species *Eubacterium rectale*, *Roseburia faecis* and *Roseburia intestinalis*. Whole grains, and especially the BR+WGB treatment, reduced plasma interleukin-6 (IL-6) and peak postprandial glucose. Shifts in the abundance of *Eubacterium rectale* were associated with changes in the glucose and insulin postprandial response. Interestingly, subjects with greater improvements in IL-6 levels harbored significantly higher proportions of *Dialister* and lower abundance of Coriobacteriaceae. In conclusion, this study revealed that a short-term intake of whole grains induced compositional alterations of the gut microbiota that coincided with improvements in host physiological measures (e.g. inflammation) related to metabolic dysfunctions in humans.

3.2 Introduction

Obesity is associated with an increased risk in cardiovascular disease, type-2 diabetes, non-alcoholic fatty-liver disease, and some cancers, and constitutes a major health concern worldwide (Hu, 2011, Cornier et al., 2008). A diet high in whole grains and dietary fibers has been shown to improve metabolic parameters related to these metabolic disorders (Fung et al., 2002, Jensen et al., 2004, Liu et al., 2009, 2003, Murtaugh et al., 2003, Nettleton et al., 2008). The mechanisms responsible for the benefits of whole grains are not completely understood. It has been proposed that the dietary fiber present in whole grains increases the viscosity of the digesta and binds to bile acids in the small intestine, thus contributing to decreased sugar and lipid (cholesterol) absorption (Behall et al., 2004, Alminger and Eklund-Jonsson, 2008). In addition, phytochemicals and other bioactive compounds in whole grains might provide metabolic benefits (Adom and Liu, 2002, Harris and Kris-Etherton, 2010, Nilsson et al., 2006). Furthermore, the metabolic inflammation associated with obesity and related diseases is now considered to trigger metabolic dysfunctions (Gregor and Hotamisligil, 2011), and the benefits of whole grains might be due to an anti-inflammatory action (Nilsson et al., 2008b, Rosén et al., 2011). In this respect, bacterial fermentation of undigestible constituents of whole grains in the gastrointestinal tract has been suggested to be partly responsible for the benefits of whole grains (Harris and Kris-Etherton, 2010, Nilsson et al., 2008a, North et al., 2009).

A consideration of the gut microbiome in the context of the health effects of whole grains has become especially relevant in the light of recent research that indicated an etiological role of gut bacteria in metabolic disorders. Obesity and type 2 diabetes have been linked to alterations in the intestinal microbiota in both humans and animal models (Cani et al., 2007, Larsen et al., 2010, Ley et al., 2006, Turnbaugh et al.,

2006, Vijay-Kumar et al., 2010). If these aberrations contribute to human disease is still unclear, but pathophysiological indicators are reduced in animal models when animals are kept germ-free or when treated with antibiotics, and manifestations of disease can be transmitted through the gut microbiota (Cani et al., 2008, Henao-Mejia et al., 2012, Ley et al., 2005, Vijay-Kumar et al., 2010). Proposed mechanisms by which the microbiota contributes to metabolic aberrations are the induction of lipolysis leading to increased fat storage (Bäckhed et al., 2007), hepatic de-novo synthesis of triglycerides (Bäckhed et al., 2004), and the alteration of bile acid metabolites with consequences to lipid metabolism in the host (Claus et al., 2011). Furthermore, the gut microbiome might exacerbate the systemic inflammation associated with obesity and related metabolic disorders (Ding et al., 2010, Hotamisligil, 2006), possibly through the induction of endotoxemia driven by lipopolysaccharide (LPS) translocation through the intestinal epithelium (Amar et al., 2008, Cani et al., 2007, 2008, Li and Hotamisligil, 2010).

The interplay between the gut microbiota and host metabolism and the ability of whole grains to affect both of these aspects suggest that one mechanism by which whole grains confer their benefits might be through a modulation of the gut microbiome. Recent research has revealed that the composition and metabolism of the gut microbiota can be modulated through prebiotics and fiber (Flint et al., 2007, Louis et al., 2007), and these carbohydrates have been shown to improve metabolic markers in experimental models (Cani et al., 2007, Neyrinck et al., 2011). Despite these encouraging findings, human studies that investigate the effects of whole grains and cereal fibers on host metabolism have neglected, until now, to characterize the gut microbiome and explore its potential contribution in mediating health improvements (Tilg and Kaser, 2011). In addition, although the effect of fiber on the gut microbiota has been recently studied in experimental animals (Neyrinck et al., 2011, Van den

Abbeele et al., 2011), information on how whole grains impact human gut microbiome composition is lacking.

The aims of this study were to characterize the impact of the incorporation of whole grains to an otherwise unrestricted diet on gut microbial ecology in healthy human subjects, and to investigate whether a connection with metabolic and immunological improvements exists. For this purpose, we performed a human crossover study with three four-week whole grain treatments, and collected fecal and blood samples at baseline and at the end of each treatment. The effect of whole grains on fecal microbiota composition was characterized by pyrosequencing of 16S rRNA gene tags, and inflammatory and metabolic markers related to metabolic dysfunctions in humans were measured in blood samples. The molecular characterization of fecal microbiota in parallel to host phenotyping allowed an investigation of associations between dietary induced metabolic changes and shifts in the gut microbiome.

3.3 Materials and methods

3.3.1 Subjects

The human trial was approved by the Institutional Review Board of the Kansas State University (KSU) (IRB Approval Number: 5298), and written informed consent was obtained from all subjects. Healthy participants (see Supplementary materials for inclusion/exclusion criteria) were recruited through leaflets distributed on-campus by the College of Human Nutrition at the Kansas State University, Manhattan, KS. Twenty-eight participants, seventeen females and eleven males (age 25.9 $\pm$ 5.5 years) took part in the study.

3.3.2 Test meals

Whole grain Prowashonupana Barley (Sustagrain®Barley Quick Flakes, ConAgra Mills) and whole grain brown rice (Insta Grains®Brown Rice Flakes, Briess) flakes were used in this study. Three test meals with different amounts of total dietary fiber (TDF) were included: a barley treatment (WGB), consisting of 60 g of barley (18.7 g TDF); a brown rice and barley treatment (BR+WGB), consisting of 30 g each barley and brown rice (11.5 g TDF); and a brown rice treatment (BR), consisting of 60 g of brown rice (4.4 g TDF). Subjects were provided with individual bags containing a daily dose of the corresponding treatment (60 g of flakes). Nutritional information of the whole grain flakes used in the study is available in the Supplementary materials and Supplementary Table 3.3.

3.3.3 Study design

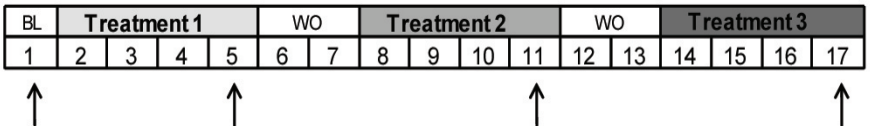

Figure 3.1: **Experimental design.** Time line of the randomized crossover trial. Three four-week dietary treatments were assessed in succession. The treatments were interspaced by two-week wash-out (WO) periods. Blood and stool samples (indicated by arrows) were collected during the baseline (BL) and at the end of each four-week treatment period.

The study was conducted as a randomized crossover trial over 17 weeks (Figure3.1). The first week served as a baseline period, after which each subject underwent three four-week dietary treatments (BR, BR+WGB, WGB) in random order, and inter-

spaced by two-week washout periods. The study was conducted under free-living conditions, and no dietary restrictions were imposed except that subjects were expected to be non-vegetarian. Subjects were instructed to consume the 60 g of flakes daily either plain, with yogurt or with milk, without time restrictions. Weekly symptom diaries were completed by the subjects in which they self-reported bowel movement, discomfort, flatulence, bloating, stool consistency and general well-being on a scale from 1 to 5 (1 being optimal/normal and 5 worst/abnormal).

3.3.4 Subject parameters and determination of metabolic and immunological markers

Subject parameters were measured at the Human Metabolism Laboratory at KSU. Total body composition was assessed at baseline with dual-energy X-ray absorptiometry (Prodigy, GE-Lunar, USA). Blood samples were drawn at baseline and at the end of each dietary treatment after a 12 h overnight fast. An initial blood sample was drawn (time 0). A standard drink containing 75 g of glucose (Fisher) was consumed within 10 min, and blood samples were collected at 15, 30, 45, 60, 90 and 120 min postprandially. Blood was immediately placed in tubes containing K2-EDTA (Vacutainer, BD, USA) and centrifuged at 1,000-1,500 x g for 13 min at 5-10°C. Aliquots of plasma were transferred into tubes for storage at -80°C until further testing.

Glucose and insulin were measured in plasma samples in duplicate using an automated analyzer (YSI 2300, USA) and the Human Gut Hormone Immunoassay kit (Milliplex, USA) with a dual laser flow cytometer (Luminex 100), respectively. A lipid profile, consisting of total cholesterol (TC), high-density lipoprotein (HDL), and non-HDL cholesterol was performed on the preprandial samples (time 0) using the Cholestech LDX System (Cholestech, USA). Three markers of inflammation were

measured in plasma samples by enzyme-linked immunosorbent assays (ELISAs) (in duplicate): lipopolysaccharide binding protein (LBP) (USCN Life Science and Technology, USA), high-sensitive C-reactive protein (hs-CRP) (Symansis, New Zealand), and interleukin 6 (IL-6) (R&D Systems, USA).

Short chain fatty acids (SCFA) were quantified in fecal samples by gas chromatography as described in the Supplementary materials.

3.3.5 Compositional analysis of the fecal microbiota by pyrosequencing

Subjects provided fecal samples within 24 h of blood sampling and 2 h of defecation. Fecal material and 1:10 fecal homogenates in phosphate buffered saline (pH=7) were immediately frozen (-80°C) and stored until further processing. Bacterial DNA was extracted from fecal homogenates as described by Martínez and co-workers (2010), using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) in combination with enzymatic and mechanical cell lysis. Pyrosequencing of amplicons obtained by PCR with universal primers targeting the V1-V3 region of the 16S rRNA gene was performed as previously described (Martínez et al., 2010), using the 454 Genome Sequencer FLX with GS FLX Titanium series reagents at the Core for Applied Genomics and Ecology (CAGE, University of Nebraska).

Sequence processing was performed combining features of QIIME (Caporaso et al., 2010) and the Ribosomal Database Project (RDP) pipeline (Cole et al., 2009). Three-thousand quality-controlled sequences per sample were randomly selected and used for taxonomic classification. Sequences were assigned to a bacterial phylum, family, and genus using the Classifier tool of the RDP (Wang et al., 2007). In addition, sequences were assigned to Operational Taxonomic Units (OTU) with 97% sequence

homology as described in the Supplementary materials. Chao1 species richness estimator, and Shannons and Simpsons (defined as 1-Dominance) diversity indices were computed with QIIME.

3.3.6 Query for genes encoding β-glucanases in genomes of human gut microbes

The web-based Integrated Microbial Genomes platform (IMG) of the Joint Genome Institute (JGI) (http://img.jgi.doe.gov/) was used to identify large-bowel associated bacteria with β-glucanase encoding activity. The strains included are listed in the Supplementary materials. For the species identified to contain β-glucanase genes, their abundance in the fecal microbiota of our subjects was quantified by BLASTn.

3.3.7 Statistics

Results are presented as means $\pm$ SD. Differences in bacterial taxa and host phenotypes among treatments were determined by one-way ANOVA with repeated measures in combination with Tukeys post-hoc tests, and $P<0.05$ was considered statistically significant. If the data were not normally distributed, values were subjected to transformations such as square root or logarithm with base 10 to achieve normality. If normality could not be achieved through transformations, non-parametric tests were performed (i.e.: Kruskall-Wallis). When only two groups of data were compared, Students t-tests were performed. Correlations between host parameters and bacterial populations were assessed by Pearsons correlation tests using GraphPad Prism version 5.00 (GraphPad Software, USA). Associations between inflammatory markers and gut microbiome composition were also analyzed through linear models using SAS. Additional information on the statistical methods can be found in the Supplementary

materials.

3.4 Results

3.4.1 Physiologic, metabolic, and microbiome characteristics of the study population

Twenty-eight volunteers, eleven males and seventeen females, participated in the nutritional trial, and subjects parameters are presented in Table 3.1. Based on percent body fat, thirteen subjects were considered overweight, using as cutoff values >31% body fat for women and >25% for men for classification as overweight. Metabolic and immunological markers included in the study were plasma fasting glucose and insulin levels, glycemic and insulin postprandial response, a lipid panel (TC, HDL, non-HDL), and inflammatory markers (hs-CRP, IL-6 and LBP). The rationale for the inclusion of these markers is their suitability in determining the progression of metabolic aberrancies and the risk of cardiovascular disease (Cardellini et al., 2005, Ridker, 2009, Schumann et al., 1990, Spranger et al., 2003), and their association with obesity (Sun et al., 2010). Accordingly, positive correlations between body fat and all three inflammatory markers were observed (Figure 3.2 A-C). LBP and hs-CRP were highly correlated ($r=0.90$, $P<0.0001$) (Figure 3.2 D). The linear model identified body fat as a significant factor affecting IL-6 ($P<0.01$), hs-CRP ($P<0.0001$), and LBP ($P<0.0001$). Furthermore, significant positive correlations existed between markers of inflammation and glucose metabolism (Figure 3.2 A). Together, these associations substantiate the link between adiposity and a low-grade systemic inflammation (Hotamisligil, 2006).

Pyrosequencing revealed that the baseline fecal microbiota of the participants was

Table 3.1: **Baseline characteristics of the 28 subjects, and differentiated by gender and percent body fat.** Values are presented as mean ± SD.

	Overall	Gender			Body fat [a]		
	All subjects	Male	Female	P-value	Overweight	Normoweight	P-value[b]
Age	25.9 ± 5.4	26.7 ± 5.4	25.4 ± 5.8	NS	28.6 ± 6.6	23.6 ± 3.0	<0.05
Weight (kg)	72.3 ± 18.3	87.7 ± 17.1	62.3 ± 10.5	<0.001	79.7 ± 19.5	65.9 ± 14.9	<0.05
BMI (kg/m^2)	25.1 ± 4.5	27.4 ± 4.8	23.6 ± 3.7	<0.05	27.9 ± 4.4	22.7 ± 3.0	<0.001
Body fat mass (kg)	20.8 ± 10.3	20.2 ± 11.6	21.2 ± 9.8	NS	29.7 ± 8.1	13.1 ± 3.6	<0.001
Body fat (%)	29.6 ± 11.0	22.8 ± 8.5	34.0 ± 10.8	<0.01	39.2 ± 7.4	21.3 ± 6.3	<0.001
Cholesterol (mmol/l)							
Total cholesterol	4.86 ± 1.12	4.07 ± 0.69	5.22 ± 1.67	<0.01	4.76 ± 1.26	4.75 ± 1.07	NS
Non high-density lipoprotein	3.13 ± 1.03	2.78 ± 0.74	3.29 ± 1.38	NS	3.23 ± 1.21	2.94 ± 0.85	NS
High-density lipoprotein	1.65 ± 0.42	1.30 ± 0.28	1.84 ± 0.57	<0.001	1.53 ± 0.45	1.65 ± 0.42	NS
Fasting plasma glucose (mmol/l)	5.17 ± 0.74	5.14 ± 0.72	5.15 ± 1.42	NS	5.44 ± 0.93	4.94 ± 0.40	NS
Fasting plasma insulin (μIU/ml)	43.44 ± 18.86	42.76 ± 20.55	44.93 ± 21.14	NS	49.77 ± 19.92	40.34 ± 18.38	NS
Inflammatory markers							
IL-6 (pg/ml)	1.75 1.43	1.18 0.81	2.01 1.60	NS	2.29 1.34	1.28 1.33	NS
(min-max)	(0.06-5.17)	(0.33-2.59)	(0.06-5.17)		(0.65-4.89)	(0.06-5.17)	
Hs-CRP (mg/L)	1.69 2.24	0.32 0.22	2.38 2.46	<0.01	2.47 2.36	0.97 1.87	NS
(min-max)	(0.002-7.039)	(0.052-0.805)	(0.002-7.039)		(0.115-6.696)	(0.002-7.039)	
LBP (μg/ml)	15.09 19.85	4.42 3.03	20.91 22.41	<0.01	23.98 24.90	7.48 8.60	NS
(min-max)	(0.12-88.05)	(1.00-9.61)	(0.12-88.05)	(2.12-88.05)	(0.12-28.51)		

[a] Women with over 31% body fat, and men with over 25% body fat were considered as overweight individuals. All others were considered lean.

[b] NS = Not significant.

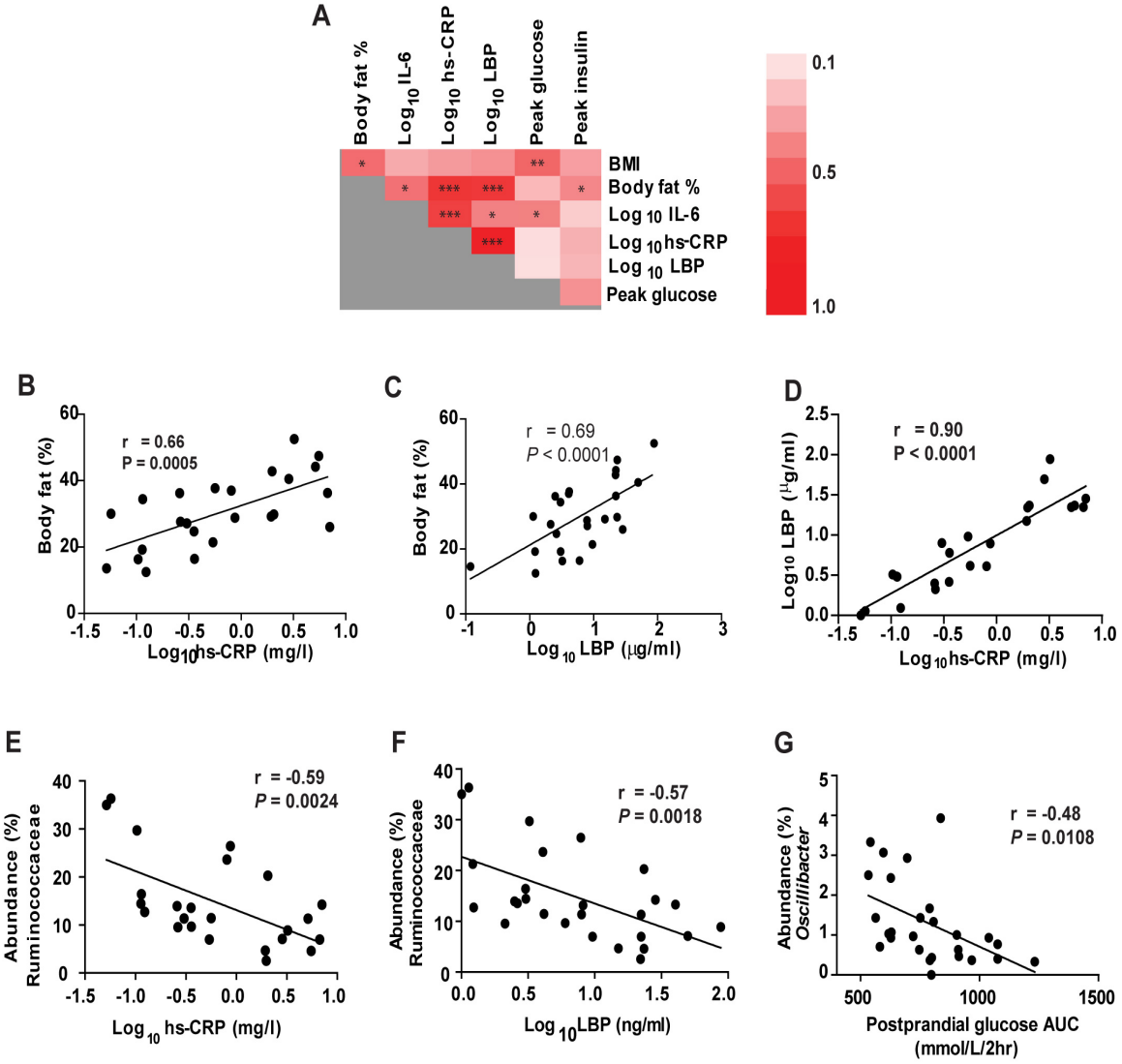

Figure 3.2: **Associations among host physiological characteristics and their correlation with bacterial populations in fecal samples at baseline.** Heatmap displaying correlation coefficients between metabolic and physiological parameters of the study population at baseline (A). Correlations between hs-CRP with body fat (B), LBP with body fat (C), hs-CRP and LBP (D), hs-CRP and Ruminococcaceae (E), LBP with Ruminococcaceae (F), and Oscillibacter with postprandial AUC glucose (F). Pearsons r correlation and the corresponding P values are presented.

dominated by the phyla Firmicutes and Bacteroidetes, with lower proportions of Verrucomicrobia and Actinobacteria, in agreement with previous molecular characterizations of the human fecal microbiota (Ley et al., 2006, Martínez et al., 2010). We investigated whether associations between host phenotypes and microbial populations existed (Supplementary Figure 3.5). No significant correlation was determined between any bacterial group and percent body fat or BMI, although overweight subjects harbored significantly lower abundances of Ruminococcaceae (10.8 ± 5.4% versus 17.9 ± 9.9%, $P<0.05$) and *Faecalibacterium* (1.8 ± 1.8% versus 3.7 ± 2.5%, $P<0.05$). The analysis revealed negative correlations between the family Ruminococcaceae and all three inflammatory markers at baseline (Figures 3.2 E-F, and Supplementary Figure 3.5). Within this family, the genera *Faecalibacterium* and *Ruminococcus* displayed negative correlations with hs-CRP ($r=-0.48$, $P<0.05$, and $r=-0.60$, $P<0.01$, respectively). The analysis also revealed a negative association between *Oscillibacter* and postprandial glucose AUC (Figure 3.2F). Regarding the markers of lipid metabolism, proportions of Bacteroidetes, Bacteroidaceae, and *Bacteroides* were positively correlated to plasma HDL values ($r=0.54$, $P<0.05$; $r=0.56$, $P<0.05$; $r=0.56$, $P<0.05$; respectively) (Supplementary Figure 3.6).

3.4.2 Supplementation of whole grains altered composition of the fecal microbiota

Sequence data determined by pyrosequencing were used to establish the effects of whole grains on the gut microbiota composition, and this analysis revealed that all three test meals impacted the gut microbiota. All three treatments significantly increased the bacterial diversity measured by Shannons and Simpsons indices but not by Chao1 (Supplementary Figure 3.7). These results indicate that although species

richness did not increase (determined by Chao1), community evenness did (Shannons and Simpsons).

Table 3.2: **Abundance of dominant bacterial taxa in fecal samples as determined by 454 pyrosequencing**. Values are presented as mean ± SD.

	Baseline	BR	BR+WGB	WGB	P-value	Confirmation by non linear model
Phylum						
Firmicutes	57.30 ± 14.13	65.06 ± 11.40†	65.53 ± 10.64†	65.42 ± 12.05†	0.003	Yes
Bacteroidetes	37.99 ± 14.35	30.74 ± 11.62†	29.85 ± 11.93†	30.32 ± 12.22†	0.01	Yes
Verrucomicrobia	1.82 ± 1.98	1.34 ± 1.53	0.68 ± 0.80	0.59 ± 0.80	NS	Yes
Actinobacteria	1.24 ± 0.97	1.42 ± 1.78	2.23 ± 3.32	2.05 ± 2.73	NS	Yes
Family						
Bacteroidaceae	28.55 ± 15.73	22.89 ± 10.37	21.19 ± 11.87†	23.48 ± 12.62	0.013	Yes
Lachnospiraceae	22.21 ± 7.90	22.62 ± 7.91	23.11 ± 6.56	22.65 ± 7.63	NS	Yes
Ruminococcaceae	14.64 ± 8.76	17.32 ± 8.90	16.53 ± 8.06	15.82 ± 8.32	NS	Yes
Incertae Sedis XIV	5.79 ± 3.15	7.63 ± 4.47	8.16 ± 3.97††	8.62 ± 4.32††	0.001	Yes
Porphyromonadaceae	3.40 ± 3.07	2.69 ± 3.42	2.76 ± 3.10	1.95 ± 1.55†	0.022	No
Prevotellaceae	2.97 ± 9.24	2.34 ± 6.56	3.59 ± 10.10	2.39 ± 6.50	NS	Yes
Verrucromicrobiaceae	1.85 ± 4.58	0.77 ± 1.53	0.68 ± 1.28	0.59 ± 0.80	NS	Yes

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Table 3.2 – continued from previous page

	Baseline	BR	BR+WGB	WGB	P-value	Confirmation by non linear model
Rikenellaceae	1.77 ± 2.09	1.68 ± 1.85	1.12 ± 1.06	1.35 ± 1.68	NS	Yes
Veillonellaceae	1.59 ± 1.13	1.52 ± 1.19	1.86 ± 1.19	1.97 ± 1.60	NS	Yes
Genus						
Bacteroides	28.55 ± 15.73	22.89 ± 10.37	21.19 ± 11.87†	23.48 ± 12.62	0.022	Yes
Blautia	5.68 ± 3.15	7.61 ± 4.47	8.14 ± 3.97††	8.61 ± 4.32††	0.001	Yes
Ruminococcus	4.20 ± 4.91	5.35 ± 5.05	4.171 ± 5.75	3.46 ± 4.32	NS	Yes
Faecealibacterium	2.82 ± 2.38	3.06 ± 2.29	3.86 ± 3.22	3.86 ± 3.19	NS	Yes
Prevotella	2.79 ± 8.89	1.99 ± 6.24	3.34 ± 9.84	2.02 ± 6.30	NS	Yes
Dorea	2.59 ± 2.01	3.12 ± 2.22	3.08 ± 1.80	2.75 ± 1.86	NS	Yes
Parabacteroides	2.58 ± 3.05	2.06 ± 3.23	2.10 ± 3.14	1.59 ± 1.44	NS	Yes
Roseburia	1.98 ± 1.35	1.70 ± 1.25	2.42 ± 1.58	3.06 ± 2.91‡‡	0.01	Yes
Akkermansia	1.85 ± 4.58	0.77 ±1.53	0.68 ± 1.28	0.59 ± 0.80	NS	Yes
Coprococcus	1.82 ± 2.09	1.91 ± 2.08	1.47 ± 2.22	1.35 ± 1.78	NS	Yes
Alistipes	1.76 ± 2.08	1.67 ± 1.85	1.11 ± 1.05	1.34 ± 1.67	NS	Yes
Oscillibacter	1.27 ± 1.04	1.24 ± 1.00	1.08 ± 0.83	0.96 ± 0.61	NS	Yes
Bifidobacterium	0.99 ± 1.88	1.02 ± 1.64	1.95 ± 3.16	1.84 ± 2.54	0.011	No

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Table 3.2 – continued from previous page

	Baseline	BR	BR+WGB	WGB	P-value	Confirmation by non linear model
Subdoligranulum	0.94 ± 1.03	1.17 ± 1.43	1.42 ± 1.73	1.09 ± 1.02	NS	Yes
Dialister	0.75 ± 1.17	0.60 ± 0.89	0.94 ± 1.21	1.14 ± 1.69	0.027	No
Odoribacter	0.26 ± 0.24	0.28 ± 0.35	0.28 ± 0.41	0.15 ± 0.18††	0.002	No
OTUs						
1737 (*Odoribacter splanicus* 99%)	0.15 ± 0.14	0.13 ± 0.18	0.15 ± 0.24	0.07 ± 0.10††	0.001	No
679 (*Eubacterium rectale* 94%)	0.25 ± 0.32	0.31 ± 0.42	0.43 ± 0.57	0.57 ± 0.63††‡‡	<0.0001	Yes
956 (*Roseburia faecis* 99%)	0.12 ± 0.17	0.06 ± 0.07	0.26 ± 0.31	0.53 ± 0.92††‡‡‡	<0.0001	Yes
770 (*Roseburia intestinalis* 100%)	0.09 ± 0.12	0.04 ± 0.05	0.17 ± 0.18	0.30 ± 0.42†‡‡‡	<0.0001	Yes
3 (*Blautia wexlerae* 100%)	1.07 ± 0.78	1.58 ± 1.11	1.49 ± 0.98	1.82 ± 1.14†††*	<0.0001	Yes
179-188 (*Blautia* spp.)	1.81 ± 1.13	2.38 ± 1.69	2.75 ± 1.75	2.80 ± 2.04††	0.006	Yes
44-19-199-93 (*Eubacterium rectale* 98%)	2.48 ± 2.67	2.75 ± 3.27	3.65 ± 3.45	4.83 ± 3.98†‡‡*	0.001	Yes

Significantly different † P<0.05 †† P<0.01 ††† P<0.001 compared to Baseline.

Significantly different ‡ P<0.05 ‡‡ P<0.01 ‡‡‡ P<0.001 compared to BR.

Significantly different * P<0.05 ** P<0.01 *** P<0.001 compared to BR+WGB.

NS = Not significant.

The analysis revealed that whole grains had a measurable effect on the composition of the gut microbiota. In accordance to previous studies that assessed the effect of diet on the gut microbiome (Davis et al., 2011, Martínez et al., 2010), substantial inter-individual variation was observed (Supplementary Table 3.4). Despite this variability, several diet induced shifts reached statistical significance in the entire study population. The proportion of the phylum Firmicutes increased, while Bacteroidetes were reduced (Table 3.2). The decrease in Bacteroidetes was largely caused by a reduction of the genus *Bacteroides* (Table 3.2).

The increase in Firmicutes was more comprehensive and shifts in the abundance of several taxa were detected. All three dietary treatments increased the abundance of the genus *Blautia* and two OTUs within this genus (Table 3.2), although significance was only achieved when WGB was included in the treatment. Several compositional shifts were strictly associated with the consumption of WGB, namely the genera *Roseburia*, *Bifidobacterium*, and *Dialister* and the species *Eubacterium rectale*, *Roseburia faecis*, and *Roseburia intestinalis* (Table 3.2), and many of these taxa increased gradually with WGB intake. The linear regression model confirmed all of these significant changes except for the species *Bifidobacterium*, and *Dialister*. Other taxa clearly responded to WGB, but due to inter-individual variation, these shifts did not reach statistical significance. For example, *Bacteroides coprocola* showed over a ten-fold increase with WGB consumption, but the species was only detected in three subjects (Supplementary Table Table 3.4). Although both whole grains led to an increase in the Firmicutes/Bacteroidetes ratio, no family or genus showed a significant increase for BR, suggesting that this test meal induced diverse alterations in the gut microbiome that are not consistent among subjects.

No significant differences were detected in the amounts of SCFA for any of the treatments. It is possible that an increase in SCFA production could not be detected in

fecal samples as most SCFA are absorbed in the gastrointestinal tract (Millet et al., 2010).

3.4.3 Distribution of β-glucanase genes in human gut microbes

WGB contains a high amount of β-glucans (14.1%), while none were detected in BR (Supplementary Table 3.3). In order to test if the ability to hydrolyze β-glucans could explain the specific shifts in the fecal microbiota induced through WGB, we investigated distribution of β-glucanase genes in 112 strains originating from the human gut. This analysis revealed that β-glucanase genes are present in a variety of gut bacterial species from a broad taxonomic range, including 10 *Bacteroides*, 4 *Bifidobacterium*, 3 *Collinsella*, 2 *Clostridium*, 2 *Coproccus*, 2 *Eubacterium*, 1 *Ruminococcus*, 2 *Roseburia*, and 1 *Akkermansia* species (Supplementary Table S2). Of these species, only *Eubacterium rectale*, *Roseburia faecis* and *Roseburia intestinalis* were significantly increased through WGB, indicating that the mere presence of β-glucanase encoding genes does not predict the changes in community composition in response to the diet.

3.4.4 Whole grain consumption improved metabolic and immunological markers

The daily consumption of 60 g of whole grains for four weeks improved immunological and metabolic markers in the human subjects. The findings for the entire study population are shown in Supplementary Table 3.5, and differentiated by gender and body fat in Supplementary Tables 3.6, 3.7, 3.8, and 3.9. A significant decrease in plasma IL-6 levels for the BR+WGB treatment versus baseline values was detected (Figure 3.3 A). Quantitatively, this reduction was highest in overweight subjects (Fig-

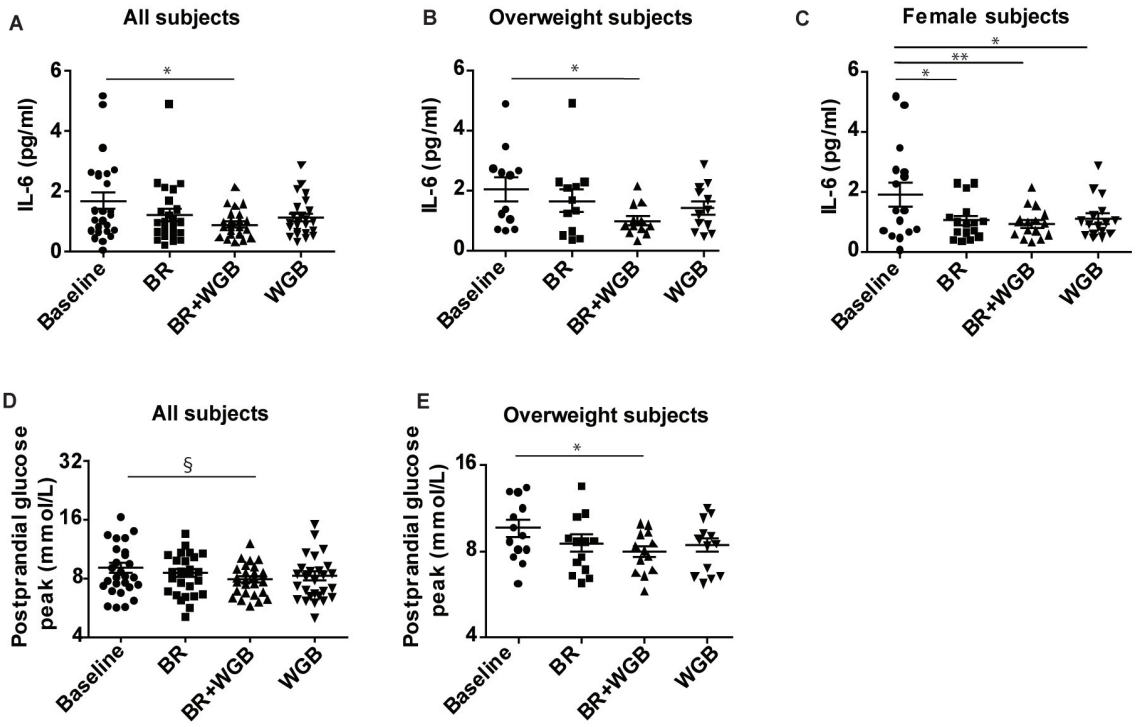

Figure 3.3: **Immunological and metabolic improvements induced through whole grain consumption.** Plasma IL-6 levels in the entire subject population (A), in overweight participants (B), and in females (C). Maximum postprandial glucose levels in the entire subject population (D) and overweight subjects (E) during the three treatments (BR, BR+WGB, WGB) and at baseline. $*$ P<0.05, $**$ P<0.01, $\oint$ P<0.1.

ure 3.3 B). In women, all three treatments significantly reduced IL-6 (Figure 3.3 C). The linear model analysis confirmed the anti-inflammatory effect of whole grains and revealed a significant reduction of IL-6 for BR+WGB and WGB treatments (P<0.01, P<0.05). Despite not achieving statistical significance due to high inter-individual variation, hs-CRP plasma levels were halved during the BR+WGB period compared to the baseline (Supplementary Tables 3.5-3.9). Whole grain consumption significantly improved glucose and lipid metabolism.

Postprandial peak glucose levels were significantly lowered in overweight subjects dur-

ing the BR+WGB period ($P<0.05$), and the reduction approached significance in the entire study population ($P<0.1$) (Figure 3.3 D-E). Fasting glucose levels were significantly decreased in women and overweight subjects, and in females, total cholesterol was significantly reduced (Supplementary Tables 3.6-3.9).

3.4.5 Improved metabolism following whole grain intake are linked to the fecal microbiota

To determine whether effects of whole grains were connected with the gut microbiome, a correlation analysis was performed between bacterial shifts and changes in metabolic markers that occurred during the BR+WGB period. We focused the analysis on the BR+WGB treatment as it induced the most significant metabolic improvements (Figure 3.3). This analysis revealed that shifts in the abundance of *Eubacterium rectale* were negatively correlated with changes in glucose AUC and insulin AUC (Supplementary Figure 3.8 A-B). The association between Eubacterium rectale and maximum postprandial glucose levels approached significance (Supplementary Figure 3.8 C).

In addition, we categorized subjects into three groups (terciles) according to the magnitude of the improvements in IL-6, hs-CRP, fasting glucose and glucose peak through BR+WGB. The baseline proportions of the bacterial groups between the three groups were compared. This analysis revealed that the gut microbiota of subjects with the highest improvement in IL-6 (3rd tercile) contained significantly higher percentages of Veillonellaceae (Figure 3.4 A), and within this family, the genus Dialister (Figure 3.4 B). Conversely, Coriobacteriaceae were significantly decreased in subjects with the highest improvement in IL-6 (Figure 3.4 C). No significant differences in microbiome composition were detected between the terciles generated for hs-CRP, fasting glucose,

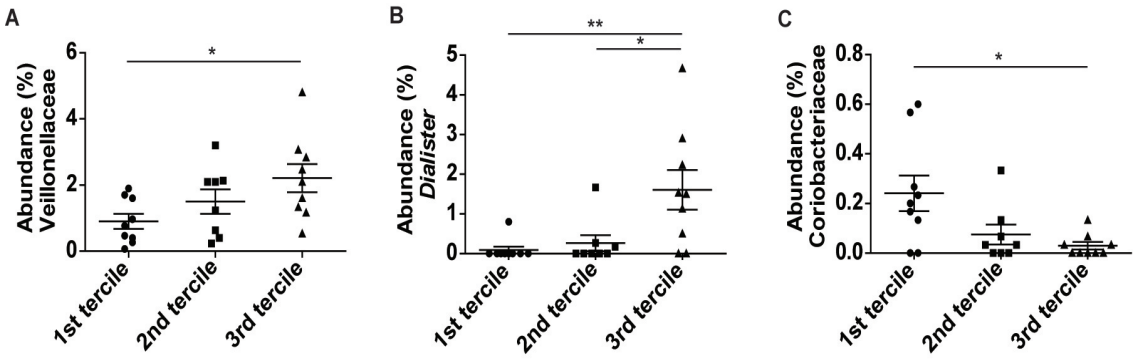

Figure 3.4: **Abundance of specific taxa in subjects that showed differences in their IL-6 response to whole grains.** Subjects were classified into terciles according to the magnitude of the change in plasma IL-6 levels induced by whole grain consumption (BR+B treatment versus baseline). The proportions of bacterial taxa in fecal samples during the baseline were compared in the three terciles and significant differences existed in the proportions of Veillonellaceae (A), *Dialister* (B) and Coriobacteriaceae (C) in fecal samples during baseline. $*$ $P<0.05$, $**$ $P<0.01$.

and postprandial glucose peak.

3.4.6 Gastrointestinal symptoms

Self-reported symptoms diaries revealed that 60 g of WGB significantly increased all the gastrointestinal symptoms surveyed, especially flatulence, while 30 g caused only a slight increase in flatulence (Supplementary Table 3.10). The addition of BR to the diet did not result in any reported changes in symptoms.

3.5 Discussion

The metabolic and immunological benefits of whole grains have been shown in various studies (Behall et al., 2004, Fung et al., 2002, Jensen et al., 2004, Nilsson et al., 2006, 2008b), and a contribution of the gut microbiome to these effects has been

suggested (North et al., 2009). However, the assessment of bacterial participation in these processes has been limited to hydrogen breath measurements, and the effects of whole grains on the gut microbiome structure have not been investigated. In this study, we showed that whole grains have a significant effect on the composition of the fecal microbiota that coincided with metabolic and immunological improvements in healthy human individuals.

All whole grain test meals caused an increase in community diversity within the subjects, driven by an increase in evenness of bacterial species. Therefore, whole grains seem to differ in their effects on the gut microbiota when compared to prebiotics and dietary fibers, which have not been shown to increase community diversity (Davis et al., 2011, Martínez et al., 2010, Van den Abbeele et al., 2011). These differences might be due to compositional complexity of whole grains, which contain an array of carbohydrates, potentially affecting a wider scope of bacterial taxa. Interestingly, a higher microbial diversity in fecal samples was also observed in children from Burkina Faso, who consumed a diet high in whole grains, legumes, and vegetables, when compared to Europeans (De Filippo et al., 2010). In addition, weaning in human infants leads to a drastic increase in diversity likely caused by the incorporation of more diverse arrays of dietary carbohydrates (reviewed in Koropatkin et al. (2012)). Therefore, it appears that bacterial diversity in the gut can be increased by providing a broader range of undigestible substrates, and our findings showed that this can be achieved by intake of whole grains.

In this study, effects on the gut microbiota composition that occurred with both BR and WGB as well as shifts specific to WGB were observed. Both whole grains increased the Firmicutes/Bacteroidetes ratio and the abundance of the genus Blautia. The overall shift in microbiota structure in favor of an expansion of Firmicutes could be the result of an increased carbohydrate intake (Duncan et al., 2008). However,

in a previous study, we did not observe an increase in the Firmicutes/Bacteroidetes ratio with the consumption of crackers containing resistant starches (Martínez et al., 2010), although the dose of carbohydrates and fiber in these crackers exceeded that of the whole grain test meals. Interestingly, a decrease of *Bacteroides* was also shown to be associated with a long-term consumption of diets rich in whole grains, dietary fibers, and vegetables in African children and US individuals (De Filippo et al., 2010, Wu et al., 2011). These and our findings suggest that other components included in whole grains and other plant derived food products might influence community structure at the phylum level, specifically decreasing Bacteroidetes. The reason for the increase in the genus *Blautia* through whole grains might be due to a syntrophic effect. *Blautia* species are acetogenic and might benefit from the production of hydrogen, which is a product of glycan fermentation, and therefore likely induced by whole grain consumption (Koropatkin et al., 2012, Nakamura et al., 2010).

We detected several bacterial taxa that displayed a specific increase with WGB, several with a clear dose response. This is likely due to its high content of β-glucans. Accordingly, the bacteria that specifically responded to WGB harbor genes encoding for β-glucanases and utilize the substrate in vitro (Hughes et al., 2008, Tasse et al., 2010). However, the in vivo findings cannot solely be explained based on functional and genomic attributes of community members, as Bacteroides species decreased during WGB consumption, but possess β-glucanase genes and can utilize β-glucans in vitro (Crittenden et al., 2002, Tasse et al., 2010, Zhao and Cheung, 2011). A possible explanation for our in vivo findings could entail preferences towards distinct β-glucan structures and molecular weights. The β(1-4) to β(1-3) linkage ratio in barley is 2.3-3, while *Bacteroides* species have been shown to especially posses β(1-3)-glucanase activity (Salyers et al., 1977). Moreover, barley derived -glucan fractions of high molecular weight have also shown little fermentation aptitude by *Bacteroides* (Hughes et al.,

2008). However, previous human trials with prebiotics and resistant starches have also revealed that the ability to utilize substrates in vitro does not predict success of species *in vivo* (Davis et al., 2011, Martínez et al., 2010, Koropatkin et al., 2012). Therefore, although the findings obtained suggest that β-glucans are the main cause for the shifts in composition induced by WGB, the exact mechanisms by which these changes are restricted to some taxa are likely to be due to competitive interactions.

A main objective of this study was to determine if the effects of whole grains on the gut microbiome are associated with physiological benefits. The whole grains in our study led to immunological and metabolic improvements, especially when BR+WGB was consumed. Plasma IL-6 was reduced, and a tendency for a decrease in plasma hs-CRP was detected. In addition to this anti-inflammatory effect, an improvement in the glycemic response during BR+WGB treatment was detected. Our findings are in agreement with previous research that established the immunological and metabolic benefits of whole grains (Casiraghi et al., 2006, Kallio et al., 2008, Nilsson et al., 2008b, Rosén et al., 2011). Most importantly, inflammation has been identified as a main cause of metabolic disorders (Hotamisligil, 2006), and the anti-inflammatory effect could provide a mechanism by which whole grains improve glucose metabolism. The anti-inflammatory effect of whole grains might be mediated through its effect on the gut microbiota. A remarkable positive correlation between LBP and hs-CRP was identified in our study population, supporting a link between bacterial LPS and systemic inflammation. The associations of these markers with body-fat support the hypothesis that endotoxemia could contribute to metabolic aberrancies (Cani et al., 2007, Delzenne and Cani, 2011). Whole grain barley caused the expansion of bacterial taxa such as bifidobacteria and *Roseburia*, which have been suggested to affect immune/inflammatory and metabolic functions in animal models (Cani et al., 2008, Neyrinck et al., 2011). Although one could envision that these shifts might under-

lie the anti-inflammatory effect of whole grains, no significant correlations between these taxa and inflammatory markers were observed. However, shifts in the abundance of *Eubacterium rectale* induced through the BR+WGB diet correlated with decreased postprandial glucose and insulin responses. This organism produces the anti-inflammatory SCFA butyrate, which might contribute to the immunological effects of whole grain consumption.

Interestingly, compositional differences at baseline were detected in the gut microbiome of subjects that differed in the magnitude of their anti-inflammatory response to whole grains. Subjects with the greatest reduction in plasma IL-6 concentration had significantly higher proportions of *Dialister* and a lower abundance of Coriobacteriaceae. These bacterial groups have been linked to chronic inflammation in previous studies. *Dialister invisus* and Coriobacteriaceae have been shown to be reduced and increased in patients with Crohns disease and colitic mice, respectively (Clavel et al., 2009, Joossens et al., 2011, Willing et al., 2010, Würdemann et al., 2009). The strong association of *Dialister* and Coriobacteriaceae with IL-6 response suggests that these taxa may condition the capability of an individual to be immunologically responsive to whole grains.

Associations between bacterial groups, inflammatory state, and host metabolism independent of whole grain intake were observed in this study during baseline (Figure 3.3). Ruminococcaceae negatively correlated with markers of inflammation and were more dominant in normoweight individuals. In addition, Bacteroidetes positively correlated with HDL cholesterol. These observations could result from an impact of these taxa on host physiology, and these associations provide a rationale to develop dietary strategies that target Ruminococcaceae and Bacteroidetes to improve human metabolic and immunological functions. However, host physiology (inflammatory state, cholesterol/bile acid metabolism) might also shape the microbiome composi-

tion. If systemic inflammation impacts levels of Ruminococcaceae and cholesterol metabolism of Bacteroidetes, then these interactions could explain the existing discrepancies related to an altered microbiome in obese versus normoweight individuals (Ley et al., 2006, Duncan et al., 2008, Schwiertz et al., 2010). Not obesity *per se*, but the associated inflammatory and metabolic aberrations could shape microbiome composition and might cause variable and more complex patterns of dysbiosis.

Our study has provided novel information about the relationship between whole grains, the gut microbiota, and host metabolism. Whole grains induced alterations in the characteristics and composition of the fecal microbiota that coincided with immunological and metabolic benefits. The ability of whole grains to increase microbial diversity in the gut might be beneficial in diseases associated with a reduced diversity (obesity, Crohns disease), and the clear associations between the reduction of IL-6 and the presence of certain bacterial taxa (*Dialister*, Coriobacteriaceae) indicate an important functional role of gut bacteria in the physiologic effects of whole grains. Although much more work is necessary to unravel the complexity of the microbiome-host interplay, the connections between the gut microbiome and diet induced physiological benefits revealed during this study represent important basic information that advocate for an increased consideration of the gut microbiota and especially its individuality in the development of personalized nutritional strategies.

3.6 Acknowledgements

Acknowledgements The dedication of the subjects is greatly appreciated. The project was supported by ConAgra Foods (Omaha, Nebraska) and matching funds through the United States Department of Agriculture, Midwest Advanced Food Manufacturing Alliance program.

3.7 Supplementary information

3.7.1 Materials and Methods

3.7.1.1 Subjects

Interviews were conducted with the volunteers to explain the protocol, determine whether they met the inclusion criteria, and record demographic data (age and gender). Exclusion criteria were treatment with antibiotics within 3 months prior to the beginning of the study or throughout its duration, being vegetarian, exercise of more than 2 h weekly, a history of a chronic gastrointestinal disorder, and the use of antihypertensive or lipid-lowering medications. Twenty-nine healthy adults were recruited to participate in this study. One female subject was excluded during the study as she required antibiotic treatment. Prior to the beginning of the study, training sessions were held to explain the protocol to the subjects.

Participants were instructed to incorporate the whole grains to their regular diet. Other instructions included withholding from strenuous physical activity and alcohol consumption on the day prior to blood drawing. Compliance with the dietary treatments was encouraged by meeting with the subjects on a weekly basis, on which occasions symptom diaries were collected and a bag with 7 daily portions of the treatment flakes were distributed.

3.7.1.2 Test meals

Prowashonupana (Sustagrain®Barley Quick Flakes, ConAgra Mills) is a waxy, hulless barley variety differing from standard barley in terms of its composition. Prowashonupana contains exceptionally high levels of total dietary fiber (30%), almost half being accounted for by -glucan, and low levels of starch (<30%). Brown rice has

high amounts of soluble starch (around 75%) and small amounts of total dietary fiber (around 7%). The processing of the barley flakes was as follows, cleaned grain kernels were roller cut and steam treated at 100.5°C for 40 min to ensure microbiological safety and passed through flaking rolls to reduce the pieces to a thickness of 0.020 ± 0.002 inches. The flakes were then cooled down to room temperature, seized, screened and packaged. The brown rice (Insta Grains®Brown Rice Flakes, Briess) was used as provided by the manufacturer. It is currently unknown how the processing conditions of both whole grains affect their functionality when compared to the unprocessed grains.

Digestible and resistant starches in the two flakes were measured in the products (K-RSTAR, Megazyme, Ireland), as well as β-glucans (K-BGLU, Megazyme, Ireland), and total dietary fiber (Andersson et al., 1999, AACC, 2011). The nutritional data of the flakes is presented in Supplementary Table 3.3.

3.7.1.3 Compositional analysis of the fecal microbiota by pyrosequencing

Sequences were binned by primer barcodes using QIIME (Caporaso et al., 2010). Sequences that were shorter than 300 bp or longer than 550 bp, contained one or more ambiguous nucleotides, had one or more mismatches to the primer or barcode, had an average quality scores below 25, or contained homopolymer runs over 6 bp, were removed. Chimeras were removed using the Blast Fragments Algorithm included in QIIME.

OTU picking was performed by aligning sequences using the RDP Infernal Alignment tool and clustered with the Complete Linkage Clustering algorithm (RDP). As current OTU picking algorithms tend to generate too many clusters (Ghodsi et al., 2007), abundance of OTUs identified to be associated with host phenotypes or di-

etary treatments were confirmed using BLASTn. For this purpose, 5 representative sequences per OTU were taxonomically assigned and aligned by ClustalW within their respective phylum. A distance matrix was generated and phylogenetic trees (one per phylum) were constructed using the Neighbor-joining algorithm (MEGA 4.0) (Tamura et al., 2007). OTUs were assigned visually as clusters within the phylogenetic trees, and membership was confirmed by sequence comparisons and restricted to sequences with >97% similarity. Consensus sequences were generated for each OTU. To quantify each OTU, a local database was created in BioEdit (Hall, 1999) with all the sequences. BLASTn with >97% similarity and >95% length overlap was used to determine the number of sequences belonging to individual OTUs. OTUs that shared a majority of their sequences were merged.

3.7.1.4 Genome queries for β-glucanase activity

The web-based Integrated Microbial Genomes (IMG) database of the Joint Genome Institute (JGI) was used to identify gut organisms with beta-glucanase function. The following bacteria were included:*Bacteroides caccae* ATCC 43185, *Bacteroides coprocola* M16, *Bacteroides dorei* 5_1_36/D4, *Bacteroides dorei* DSM 17855, *Bacteroides eggerthii* 1_2_48FAA, *Bacteroides eggerthii* DSM 20697, *Bacteroides finegoldii* DSM 17565, *Bacteroides fragilis* 3_1_12, *Bacteroides fragilis* 638R, *Bacteroides fragilis* NCTC 9343, *Bacteroides fragilis* YCH46, *Bacteroides intestinalis* 341, *Bacteroides ovatus* 3_8_47FAA, *Bacteroides ovatus* ATCC 8483, *Bacteroides ovatus* SD CC 2a, *Bacteroides ovatus* SD CMC 3f, *Bacteroides stercoris* ATCC 43183, *Bacteroides thetaiotaomicron* VPI-5482, *Bacteroides uniformis* ATCC 8492, *Bacteroides vulgatus* ATCC 8482; *Bacteroides vulgatus* PC510, *Bacteroides xylanisolvens* SD CC 1b, *Bacteroides xylanisolvens* XB1A, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium adolescentis* L2-32, *Bifidobacterium catenulatum* DSM 16992, *Bifi-*

dobacterium longum DJO10A, *Bifidobacterium longum* NCC2705, *Bifidobacterium longum* subps. *infantis* 157F-NC, *Bifidobacterium longum* subps. *infantis* ATCC 16697, *Bifidobacterium longum* subps. *infantis* JCM 1217, *Bifidobacterium longum* subsp. *longum* ATCC 55813, *Bifidobacterium longum* subsp. *longum* BBMN68, *Bifidobacterium longum* subsp. *longum* CCUG 52486, *Bifidobacterium longum* subsp. *longum* F8, *Bifidobacterium longum* subsp. *longum* JDM301, *Bifidobacterium longum* subsp. *longum* KACC 91563, *Bifidobacterium pseudocatenulatum* DSM 20438, *Blautia hansenii* VPI C7-24, *Blautia hydrogenotrophica* DSM 10507, *Bryantella formatexigens* I-52, *Butyrivibrio crossotus* DSM 2876, Clostridiales sp. SM4/1, Clostridiales sp. 1_7_47FAA, Clostridiales sp. SS3/4, Clostridiales sp. SSC/2, *Clostridium bolteae* ATCC BAA-613, *Clostridium butyricum* 5521, *Clostridium butyricum* E4, *Clostridium leptum* DSM 753, *Clostridium ramosum* VPI 0427, *Clostridium* sp. M62/1, *Clostridium spiroforme* DSM 15579, *Collinsella aerofaciens* ATCC 25986, *Collinsella intestinalis* DSM 13280, *Collinsella stercoris* DSM 13279, *Coprococcus comes* ATCC 27758, *Coprococcus eutactus* ATCC 27759, *Dialister invisus* DSM 15470, *Dorea formicigenerans* ATCC 27755, *Dorea longicatena* DSM 13814, *Eggerthella lenta* VPI 0255, *Enterococcus fecalis* ATCC 29200, *Enterococcus fecalis* ATCC 4200, *Eubacterium biforme* DSM3989, *Eubacterium cylindroides* T2-87, *Eubacterium eligens* ATCC 27750, *Eubacterium hallii* DSM 3353, *Eubacterium limosum* KIST612, *Eubacterium rectale* ATCC 33656, *Eubacterium rectale* DSM 17629, *Eubacterium rectale* M104/1, *Eubacterium ventriosum* ATCC 27560, *Faecalibacterium prausnitzii* KLE1255, *Faecalibacterium prausnitzii* A2-165, *Faecalibacterium prausnitzii* L2-6, *Faecalibacterium prausnitzii* M21/2, *Faecalibacterium prausnitzii* SL3/3, Lachnospiraceae 1_1_57FAA, Lachnospiraceae 1_4_56FAA, Lachnospiraceae 2_1_46FAA, Lachnospiraceae 2_1_58FAA, Lachnospiraceae 3_1_46FAA, Lachnospiraceae 3_1_57FAA, Lachnospiraceae 4_1_37FAA, Lachnospiraceae 5_1_37FAA, Lachnospiraceae 6_1_63FAA, Lachnospiraceae 9_1_43BFAA,

Lachnospiraceae sp 5_1_63FAA, Lachnospiraceae 8_1_57FAA, *Olsenella uli* DSM 7084, *Odoribacter splanchnicus* DSM 20712, *Parabacteroides distasonis* ATCC 8503, *Parabacteroides merdae* ATCC 43184, *Parabacteroides* sp. D13, *Phascolarctobacterium* sp YIT 12067, *Prevotella bryantii* B14, *Roseburia intestinalis* L1-82, *Roseburia intestinalis* M50/1, *Roseburia intestinalis* XB6B4, *Roseburia inulinivorans* DSM 16841, *Ruminococcaceae bacterium* D16, *Ruminococcus bromii* L2-63, *Ruminococcus gnavus* ATCC 29149, *Ruminococcus lactaris* ATCC 29176, *Ruminococcus obeum* A2-162, *Ruminococcus obeum* ATCC 29174, *Ruminococcus torques* ATCC 27756, *Ruminococcus torques* L2-14, *Slackia exigua* ATCC 700122, *Slackia heliotrinireducens* DSM 20476, *Turicibacter sanguinis* PC909.

3.7.1.5 Short chain fatty acid determination

SCFAs were determined based on approaches described by Campbell et al. (1997), with slight modifications. Undiluted fecal samples were removed from storage at -80°C and thawed on ice, and 0.4 g were diluted in 2.8 ml water containing 5-10 mM 4-methylvaleric acid and vortexed. 0.4 ml of 25% (w/v) metaphosphoric acid was added and the sample was vortexed again, followed by centrifugation for 20 min at 15,000 x *g*. The supernatant was stored overnight at -20°C. Samples were thawed and centrifuged in the same conditions as before. SCFA were quantified by gas chromatography (Perkin Elmer Clarus with Perkin Elmer Elite-FFAP column) in a 4 μl injection volume, and the data was analyzed with appropriate software (TotalChrom, Perkin Elmer, USA). Moisture quantification in the fecal samples was done as follows. Approximately 0.2 g of feces was introduced into a plastic tube with a small perforation in its cap and frozen overnight at -20°C. Samples were freeze dried for at least 36 hours until stable weight of the sample was achieved, and dry weight was calculated. SCFA were expressed on a dry basis.

3.7.1.6 Statistics

Correlations between host parameters and bacterial populations were assessed by Pearsons correlation test (GraphPad Prism v5.0). Graphs were generated for parameters that showed significant correlations and were visually inspected. If the removal of one single data-point caused the association to become non-significant, the data point was considered an outlier and removed. Associations between inflammatory markers and members of the gut microbiome were further analyzed with the following linear models:

$I_{ijt} = \beta_0 + \beta_1 \text{Fat} + \beta_2 \text{Gender} + \beta_3 \text{Age} + \beta_4 T_2 + \beta_5 T_3 + \beta_6 T_4$ (1)

$M_{hjt} = \beta_0 + \beta_1 \text{Fat} + \beta_2 \text{Gender} + \beta_3 \text{Age} + \beta_4 T_2 + \beta_5 T_3 + \beta_6 T_4$ (2)

hjt is the inflammatory marker h for subject j in treatment t, h=1,,80; j=128; t=1,2,3,4;

Fat indicates the percent body fat; Gender is a binary variable that takes values of 0 if the subject is female and 1 otherwise; Age is the age of subject in years; T2 is a binary variable that assigns 1 if the treatment is 30 g of WGB and BR each and 0 otherwise; T3 is a binary variable that assigns 1 if the treatment is 60 g of WGB and 0 otherwise; T4 is a binary variable that assigns 1 if the treatment is 60 g of BR and 0 otherwise; and T1 represents no treatment and is left out of the models as the base. Fixed effects and random effects methods were used to estimate models (1) and (2). Chi-square estimates that measure the heterogeneity of the responses clustered by subject, were used as the criterion for choice between fixed and random effects estimation methods. For the models with Chi-square values associated with $P<0.1$, random effects method was chosen.

Because hs-CRP concentrations >10 mg/l in plasma are indicative of acute inflammation unrelated to cardiovascular disease risk (Pearson et al., 2003). Therefore, 4

samples from 4 different subjects were excluded from the analysis. If the same samples also displayed abnormally high values of LBP or IL-6 levels, these data points were also considered outliers and removed. 2 and 3 samples were excluded from LBP and IL-6 analysis, respectively. One subject was excluded from the analysis of glucose parameters as incomplete data was obtained for this subject.

Table 3.3: **Nutritional information of the barley and brown rice flakes used in the study.**[a]

	Barley	Brown rice
Calories (kcal per 100g)	392	366
Fat (%)	6.7	3.0
Saturated fat (%)	1.7	1.0
Cholesterol (%)	0.0	0.0
Total carbohydrates (%)	64.6	80.0
Digestible starch (%)[b]	32.3	83.3
Resistant starch (%)[b]	0.2	0.5
Total dietary fiber (%)[c]	31.1	7.3
Insoluble fiber (%)[c]	22.8	6.8
Soluble fiber (%)[c]	8.3	0.5
β-glucan (%)[d]	14.1	0.0
Protein (%)	18.2	8.0

[a] Nutrient composition as provided by the manufacturers except when specifically noted.
[b] Measured with K-RSTAR Megazyme kit. (Expressed as dry basis).
[c] Measured according to AACCI Approved Method 32-25.01 with modifications from Andersson et al. (1999). (Expressed as dry basis).
[d] Measured with K-BGLU Megazyme kit. (Expressed as dry basis).

Table 3.4: **Human colonic bacteria with beta-glucanse enconding genes determined in the Integrated Microbial Genomes system (IMG)**. The number and type of -glucanases are indicated for the individual species. The number of subjects in which the species was detected and the direction of the shifts in response to WGB intake are presented. Proportions of the species according to dietary treatment are presented as mean ± SD.

Bacterial species	Number and type of enzymes encoded	Number of subjects in which detected	Response in individual subjects	Baseline	BR	BR+WGB	WGB	P-value
Akkermansia muniziphila	2β-glucanase precursor	10	10 NP	0.84 ± 1.82	0.57 ± 1.3	0.34 ± 0.86	0.41 ± 0.66	NS
Bacteroides caccae	7β-glucanase/β-glucanase synthase	10	17 NP	0.2 ± 0.75	0.09 ± 0.21	0.13 ± 0.31	0.13 ± 0.43	NS
Bacteroides coprocola	4 endoglucanase, 2β-glucanase/β-glucanase synthase	24	2↑, 22 NP	0.37 ± 1.52	0.14 ± 0.5	1.06 ± 3.73	1.24 ± 4.68	NS
Bacteroides dorei	1β-glucanase, 2β-glucanase/β-glucanase synthase	24	24 NP	1.88 ± 3.81	1.60 ± 2.90	1.37 ± 2.75	1.34 ± 2.84	NS
Bacteroides finegoldii	2β-glucanase/β-glucanase synthase	4	4 NP	0.04 ± 0.2	0.06 ± 0.19	0.06 ± 0.33	0.02 ± 0.08	NS
Bacteroides fragilis	2β-glucanase precursor, 3 putative β-glucanase precursor	18	1↓, 17 NP	2.68 ± 8.12	1.81 ± 6.04	1.60 ± 5.05	1.21 ± 4.56	NS

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Table 3.4 – continued from previous page

Bacterial species	Number and type of enzymes encoded	Number of subjects in which detected	Response in individual subjects	Baseline	BR	BR+WGB	WGB	P-value
Bacteroides intestinalis	2β-glucanase/β-glucanase synthase, 6 endoglucanase	25	3↑	0.32 ± 0.55	0.93 ± 2.66	0.38 ± 0.69	0.47 ± 0.76	NS
Bacteroides ovatus	2β-glucanase/β-glucanase synthase, 6 endoglucanase	ND						
Bacteroides thetaiotaomicron	3β-glucanase/precursor, 2 endoglucanase E precursor	25	25 NP	0.57 ± 0.79	0.72 ± 1.38	0.52 ± 0.98	0.40 ± 0.52	NS
Bacteroides uniformis	1β-glucanase/β-glucanase synthase, 8 endoglucanase	26	3↓, 1↑, 22 NP	4.55 ± 4.55	3.34 ± 3.3	2.77 ± 3.38	3.57 ± 4.59	NS
Bacteroides eggerthii	2 endoglucanase	7	1↓, 6 NP	0.35 ± 1.39	0.36 ± 1.03	0.32 ± 1.15	0.33 ± 0.97	NS
Blautia wexlerae	No matches found	28	6↑, 1↓, 11 NP	1.07 ± 0.78	1.58 ± 1.11	1.49 ± 0.98	1.82 ± 1.14	<0.0001
Blautia hydrogenotrophica	No matches found	4	4 NP	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.02	0.00 ± 0.01	NS
Blautia coccoides	No matches found	7	7 NP	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.02	NS

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Table 3.4 – continued from previous page

Bacterial species	Number and type of enzymes encoded	Number of subjects in which detected	Response in individual subjects	Baseline	BR	BR+WGB	WGB	P-value
Blautia producta	No matches found	4	4 NP	0.01 ± 0.03	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	NS
Blautia hansenii	No matches found	5	5 NP	0.10 ± 0.26	0.05 ± 0.16	0.18 ± 0.91	0.08 ± 0.34	NS
Blautia spp. (*Ruminococcus obeum*)	1,3-β-glucosidase	14	4↑, 10 NP	1.81 ± 1.13	2.38 ± 1.69	2.75 ± 1.75	2.80 ± 2.04	0.006
Bifidobacterium adolescentis	2 putative β-1,3-endoglucanase, 2 endoglucanase	14	2↑, 12 NP	0.22 ± 0.42	0.36 ± 0.83	0.64 ± 1.3	0.48 ± 1.08	NS
Bifidobacterium angulatum	2 endoglucanase	ND						
Bifidobacterium longum	1 putative β-1,3-exoglucanase, 2 endoglucanase	17	1↑, 16 NP	0.16 ± 0.42	0.17 ± 0.50	0.18 ± 0.33	0.23 ± 0.50	NS
Bifidobacterium pseudocatenulatum	4 endoglucanse	7	7 NP	0.07 ± 0.22	0.14 ± 0.65	0.08 ± 0.24	0.07 ± 0.20	NS
Clostridium butyricum	7 endoglucanase	4	4 NP	0.01 ± 0.03	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	NS

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Table 3.4 – continued from previous page

Bacterial species	Number and type of enzymes encoded	Number of subjects in which detected	Response in individual subjects	Baseline	BR	BR+WGB	WGB	P-value
Clostridium ramosum	2β-glucanase/β-glucanase synthase, 2 endoglucanase	ND						
Collinsella aerofaciens	2 endoglucanase	17	17 NP	0.08 ± 0.22	0.14 ± 0.28	0.1 ± 0.19	0.08 ± 0.18	NS
Collinsella intestinalis	2 endoglucanase	ND						
Collinsella stercoris	2 endoglucanase	ND						
Coprococcus comes	2 endoglucanase	25	1↑, 24 NP	0.29 ± 0.38	0.35 ± 0.42	0.33 ± 0.48	0.29 ± 0.45	NS
Coprococcus eutactus	1β-glucanase/β-glucanase synthase, 8 endoglucanase	13	1↑, 12 NP	0.68 ± 1.23	0.64 ± 1.23	0.62 ± 1.29	0.75 ± 1.35	NS
Dialister invisus	No matches found			0.52 ± 0.97	0.41 ± 0.72	0.56 ± 0.86	0.81 ± 1.41	NS
Eubacterium eligens	1 putative endoglucanase	14	2↑, 12 NP	0.22 ± 0.42	0.36 ± 0.83	0.64 ± 1.3	0.48 ± 1.08	NS
Eubacterium rectale	1 endo-1,4-β-glucanase	28	14↑, 14 NP	2.48 ± 2.67	2.75 ± 3.27	3.65 ± 3.45	4.83 ± 3.98	0.001
Roseburia inulinivorans	1 endo-1,4-β-glucanase	28	1↑, 27 NP	0.25 ± 0.3	0.21 ± 0.32	0.14 ± 0.24	0.16 ± 0.18	NS

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Table 3.4 – continued from previous page

Bacterial species	Number and type of enzymes encoded	Number of subjects in which detected	Response in individual subjects	Baseline	BR	BR+WGB	WGB	P-value
Roseburia faecis	Not in database	27	10↑, 17 NP	0.12 ± 0.17	0.06 ± 0.07	0.26 ± 0.31	0.53 ± 0.92	<0.0001
Roseburia intestinalis	5 endo-1,4-β-glucanase	28	9↑, 19 NP	0.09 ± 0.12	0.04 ± 0.05	0.17 ± 0.18	0.30 ± 0.42	<0.0001

NP = No pattern. NS = Not significant. ND = Not detected.

Table 3.5: **Treatment effect on metabolic and immunological markers for all subjects.** Metabolic data of the 28 participants, at baseline and at the end of the 4-week dietary treatments (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Overall				
	Baseline	BR	BR+WGB	WGB	P-value
Cholesterol					
Total cholesterol (mmol/l)	4.86 ± 1.15	4.76 ± 0.79	4.56 ± 0.89	4.89 ± 0.94	NS
Non-HDL (mmol/l)	3.09 ± 1.04	3.15 ± 0.84	3.00 ± 0.85	3.32 ± 0.94	NS
HDL (mmol/l)	1.63 ± 0.43	1.60 ± 0.37	1.55 ± 0.45	1.57 ± 0.36	NS
Plasma glucose					
Fasting (mmol/l)	5.15 ± 0.73	4.87 ± 0.49	4.81 ± 0.39	4.81 ± 0.50	NS
AUC ([mmol/l]^2)	784 ± 184	763 ± 164	746 ± 132	770 ± 179	NS
Max. peak (mmol/l)	9.08 ± 2.78	8.58 ± 2.02	7.92 ± 1.46	8.19 ± 2.35	<0.1
Plasma insulin					
Fasting (μUI/ml)	6.77 ± 1.96	6.60 ± 2.13	6.51 ± 2.02	7.03 ± 2.07	NS
AUC ([μUI/ml]^2)	3463 ± 1523	3606 ± 1520	3333 ± 1035	3540 ± 1481	NS
Max. peak (μUI/ml)	44.08 ± 19.19	44.70 ± 19.56	42.86 ± 14.49	45.13 ± 21.61	NS
Inflammatory markers					
IL-6 (pg/ml)	1.68 ± 1.36	1.21 ± 0.99	0.90 ± 0.45[a]	1.12 0.63	0.0295
Hs-CRP (mg/L)	1.60 ± 2.23	1.33 ± 1.65	0.95 ± 1.23	1.36 ± 1.88	NS
LBP (μg/ml)	14.41 ± 19.65	14.39 ± 2.09	13.23 ± 19.04	13.78 ± 18.30	NS

[a] P<0.05 compared to Baseline.

Table 3.6: **Treatment effect on metabolic and immunological markers for male subjects.** Metabolic data of male participants, at baseline and at the end of the 4-week dietary treatments (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Males				
	Baseline	BR	BR+WGB	WGB	P-value
Cholesterol					
Total cholesterol (mmol/l)	4.42 ± 1.11	4.59 ± 0.85	4.31 ± 0.91	4.46 ± 0.89	NS
Non-HDL (mmol/l)	2.78 ± 0.74	3.01 ± 0.96[a]	2.90 ± 0.93	3.08 ± 0.82	0.0327
HDL (mmol/l)	1.30 ± 0.28	1.41 ± 0.30	1.33 ± 0.31	1.23 ± 0.24	NS
Plasma glucose					
Fasting (mmol/l)	5.14 0.72	5.10 ± 0.64	4.91 ± 0.39	4.86 ± 0.34	NS
AUC ([mmol/l]^2)	860 ± 232	851 ± 143	762 ± 166	857 ± 180	NS
Max. peak (mmol/l)	10.13 ± 3.25	10.08 ± 1.67	8.21 ± 1.80	8.99 ± 2.37	<0.1
Plasma insulin					
Fasting (μUI/ml)	6.63 ± 1.75	5.93 ± 1.90	6.38 ± 1.80	6.05 ± 2.12	NS
AUC ([μUI/ml]^2)	3436 ± 1787	3816 ± 1704	3399 ± 1086	3600 ± 1586	NS
Max. peak (μUI/ml)	42.76 ± 20.55	48.63 ± 19.14	41.46 ± 12.37	48.54 ± 22.37	NS
Inflammatory markers					
IL-6 (pg/ml)	1.18 ± 0.81	1.42 ± 1.35	1.99 ± 3.63	1.09 ± 0.58	NS
Hs-CRP (mg/L)	0.35 ± 0.22	0.92 ± 1.26	0.31 ± 0.24	0.76 ± 1.07	NS
LBP (μg/ml)	4.76 ± 2.96	6.50 ± 5.48	4.42 ± 2.22	6.19 ± 4.09	NS

[a] P<0.05 compared to Baseline.

Table 3.7: **Treatment effect on metabolic and immunological markers for female subjects.** Metabolic data of female participants, at baseline and at the end of the 4-week dietary treatments (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Females				
	Baseline	BR	BR+WGB	WGB	P-value
Cholesterol					
Total cholesterol (mmol/l)	5.02 ± 1.14	4.87 ± 0.76	4.73 ± 0.87	5.15 ± 0.89	0.0342
Non-HDL (mmol/l)	3.29 ± 1.16	3.24 ± 0.78	3.06 ± 0.83	3.47 ± 1.00	NS
HDL (mmol/l)	1.84 ± 0.37	1.73 ± 0.36	1.69 ± 0.48	1.76 ± 0.25	NS
Plasma glucose					
Fasting (mmol/l)	5.15 ± 0.76	4.72 ± 0.29	4.75 ± 0.40	4.77 ± 0.59	0.0344
AUC ([mmol/l]^2)	739 ± 138	706 ± 155	735 ± 110	718 ± 162	NS
Max. peak (mmol/l)	8.40 ± 2.27	7.61 ± 1.61	7.74 ± 1.23	7.66 ± 2.26	NS
Plasma insulin					
Fasting (μUI/ml)	6.85 ± 2.12	7.04 ± 2.22	6.60 ± 2.20	7.66 ± 1.83	NS
AUC ([μUI/ml]^2)	3480 ± 1405	3483 ± 1442	3294 ± 1037	3505 ± 1465	NS
Max. peak (μUI/ml)	44.93 ± 18.86	42.15 ± 19.98	43.76 ± 16.01	42.92 ± 21.50	NS
Inflammatory markers					
IL-6 (pg/ml)	2.01 ± 1.58	1.16 ± 0.83[a]	1.10 ± 0.86[b]	1.67 ± 2.39[a]	0.0028
Hs-CRP (mg/L)	2.35 ± 2.56	1.57 ± 1.85	1.33 ± 1.43	1.72 ± 2.19	NS
LBP (μg/ml)	20.44 ± 23.19	19.32 ± 25.62	18.73 ± 22.74	18.52 ± 22.04	NS

[a] P<0.05 compared to Baseline.
[b] P<0.01 compared to Baseline.

Table 3.8: **Treatment effect on metabolic and immunological markers for overweight subjects.** Metabolic data of overweight participants, at baseline and at the end of the 4-week dietary treatments (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Overweight				
	Baseline	BR	BR+WGB	WGB	P-value
Cholesterol					
Total cholesterol (mmol/l)	4.84 ± 1.26	4.84 ± 0.86	4.51 ± 0.89	5.03 ± 1.05	NS
Non-HDL (mmol/l)	3.24 ± 1.21	3.35 ± 0.97	3.12 ± 0.90	3.52 ± 1.05	NS
HDL (mmol/l)	1.61 ± 0.45	1.47 ± 0.32	1.37 ± 0.40	1.51 ± 0.35	NS
Plasma glucose					
Fasting (mmol/l)	5.37 ± 0.93	4.87 ± 0.45[a]	4.88 ± 0.37	4.88 ± 0.42	0.0231
AUC ([mmol/l]^2)	867 ± 184	800 ± 187	774 ± 128	811 ± 160	NS
Max. peak (mmol/l)	9.66 ± 2.14	8.53 ± 2.11	7.99 ± 1.32[a]	8.43 ± 1.79	0.0428
Plasma insulin					
Fasting (μUI/ml)	6.93 ± 1.70	7.10 ± 2.53	6.82 ± 1.78	7.60 ± 1.58	NS
AUC ([μUI/ml]^2)	3730 ± 1677	3952 ± 1665	3249 ± 1128	3804 ± 1482	NS
Max. peak (μUI/ml)	48.39 ± 19.92	50.23 ± 22.63	43.19 ± 16.16	49.94 ± 21.17	NS
Inflammatory markers					
IL-6 (pg/ml)	2.03 ± 1.32	1.64 ± 1.27	0.97 ± 0.52[a]	1.40 ± 0.77	0.0438
Hs-CRP (mg/L)	2.26 ± 2.47	2.12 ± 1.96	1.37 ± 1.52	1.86 ± 1.87	NS
LBP (μg/ml)	22.45 ± 24.90	23.56 ± 26.42	21.63 ± 23.90	22.16 ± 22.66	NS

[a] $P<0.05$ compared to Baseline.

Table 3.9: **Treatment effect on metabolic and immunological markers for normoweight subjects.** Metabolic data normoweight participants, at baseline and at the end of the 4-week dietary treatments (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Normoweight				
	Baseline	BR	BR+WGB	WGB	P-value
Cholesterol					
Total cholesterol (mmol/l)	4.75 ± 1.07	4.69 ± 0.75	4.61 ± 0.92	4.77 ± 0.84	NS
Non-HDL (mmol/l)	2.94 ± 0.85	2.98 ± 0.71	2.89 ± 0.83	3.14 ± 0.83	NS
HDL (mmol/l)	1.65 ± 0.42	1.72 ± 0.38	1.71 ± 0.45	1.62 ± 0.37	NS
Plasma glucose					
Fasting (mmol/l)	4.94 ± 0.40	4.87 ± 0.54	4.75 ± 0.42	4.74 ± 0.57	NS
AUC ([mmol/l]^2)	707 ± 153	730 ± 140	720 ± 135	731 ± 192	NS
Max. peak (mmol/l)	8.58 ± 3.09	7.86 ± 1.62	7.98 ± 2.80	8.63 ± 2.01	NS
Plasma insulin					
Fasting (μUI/ml)	6.62 ± 2.22	6.17 ± 1.69	6.24 ± 2.24	6.54 ± 2.36	NS
AUC ([μUI/ml]^2)	3216 ± 1382	3284 ± 1354	3411 ± 978	3295 ± 1493	NS
Max. peak (μUI/ml)	40.34 ± 18.38	39.90 ± 15.67	42.57 ± 13.45	40.96 ± 26.83	NS
Inflammatory markers					
IL-6 (pg/ml)	1.35 ± 1.36	0.81 ± 0.32	0.83 ± 0.38	0.86 ± 0.32	NS
Hs-CRP (mg/L)	1.04 ± 1.93	0.66 ± 0.99	0.59 ± 0.83	0.94 ± 1.86	NS
LBP (μg/ml)	6.36 ± 6.67	4.83 ± 5.58	5.40 ± 5.63	5.21 ± 6.83	NS

[a] P<0.05 compared to Baseline.
[b] P<0.01 compared to Baseline.

Table 3.10: **Gastrointestinal symptoms.** Weekly gastrointestinal symptoms of the 28 participating subjects, scored in a scale from 1 (best/normal) to 5 (worst/abnormal) during the baseline and at the end of each 4-week dietary treatment (BR, BR+WGB, WGB). Values are presented as mean ± SD.

	Baseline	BR	BR+WGB	WGB	P-value
Bowel movement	1.5 ± 0.5	1.3 ± 0.5	1.7 ± 0.6	2.0 ± 0.8[a,d]	<0.01
Stool consistency	1.5 ± 0.6	1.4 ± 0.5	1.8 ± 0.6	2.0 ± 0.8[a,d]	<0.01
General well-being	1.2 ± 0.3	1.2 ± 0.4	1.5 ± 0.6	2.2 ± 0.6[b,e,g]	<0.001
Flatulence	1.3 ± 0.5	1.4 ± 0.5	2.2 ± 0.9[b,e]	3.1 ± 1.0[b,g,h]	<0.001
Abdominal pain	1.1 ± 0.2	1.1 ± 0.3	1.4 ± 0.6	1.8 ± 0.8[c,e,f]	<0.001
Bloating	1.2 ± 0.4	1.2 ± 0.4	1.6 ± 0.7[a,c]	2.2 ± 0.8[b,e,g]	<0.001

[a] P<0.05 compared to Baseline.
[b] P<0.001 compared to Baseline.
[c] P<0.05 compared to BR.
[d] P<0.01 compared to BR.
[e] P<0.001 compared to BR.
[f] P<0.05 compared to BR+B.
[g] P<0.01 compared to BR+B.
[h] P<0.001 compared to BR+B.

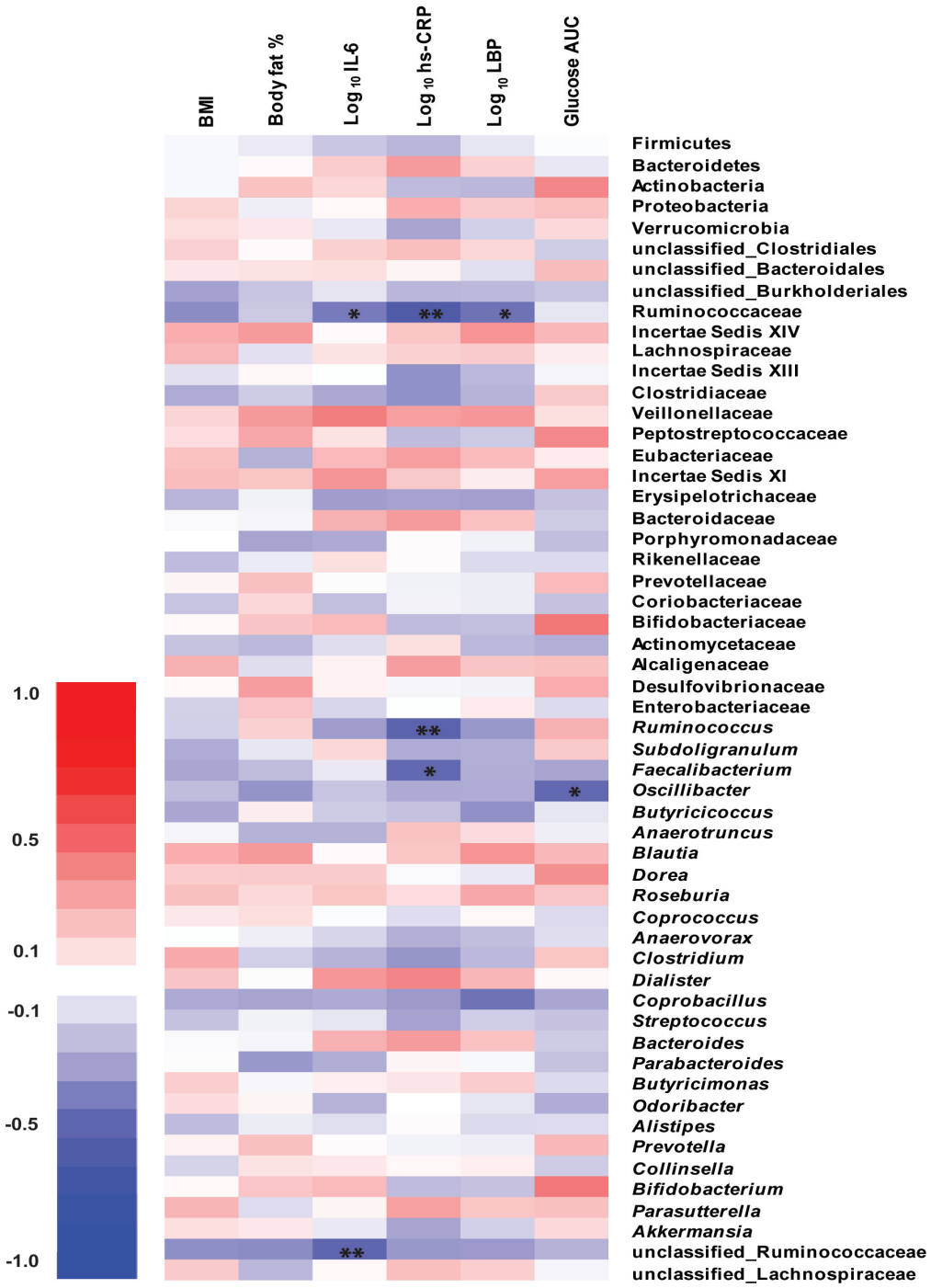

Figure 3.5: **Association between inflammatory and metabolic markers and bacterial taxa in fecal samples.** A heat map shows correlation coefficients (Pearson) between BMI, percent body fat, IL-6, hs-CRP, LBP and glucose AUC with proportions of bacterial taxa in fecal samples.

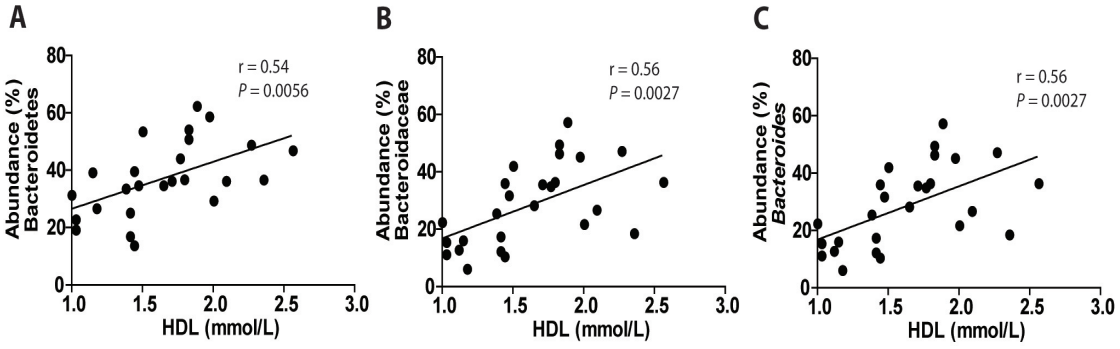

Figure 3.6: **Associations between Bacteroidetes related taxa and HDL plasma levels at baseline.** Correlations between proportions of Bacteroidetes (A), Bacteroidaceae (B) and *Bacteroides* (C) in fecal samples with HDL measured in plasma at baseline. Pearsons r correlation and the P values are presented.

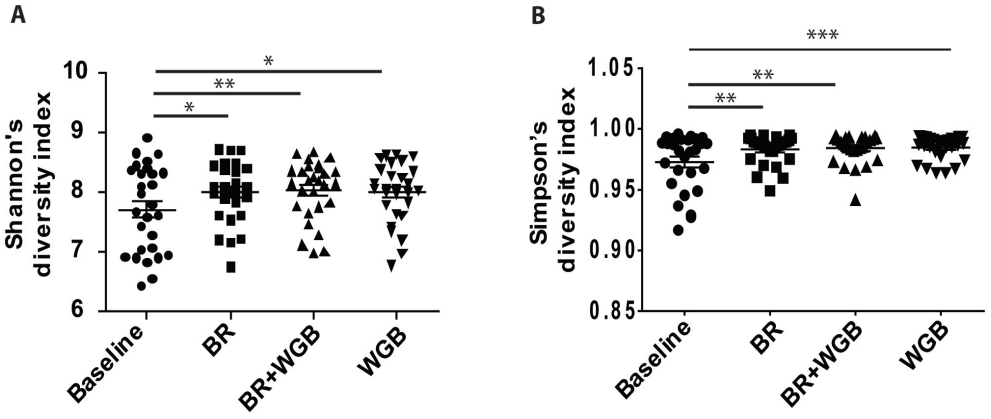

Figure 3.7: **Impact of whole grains on the fecal microbiota.** Diversity of the bacterial population in fecal samples assessed by Shannons (A) and Simpsons (B) -diversity indices. $*$ P<0.05, $**$ P<0.01.

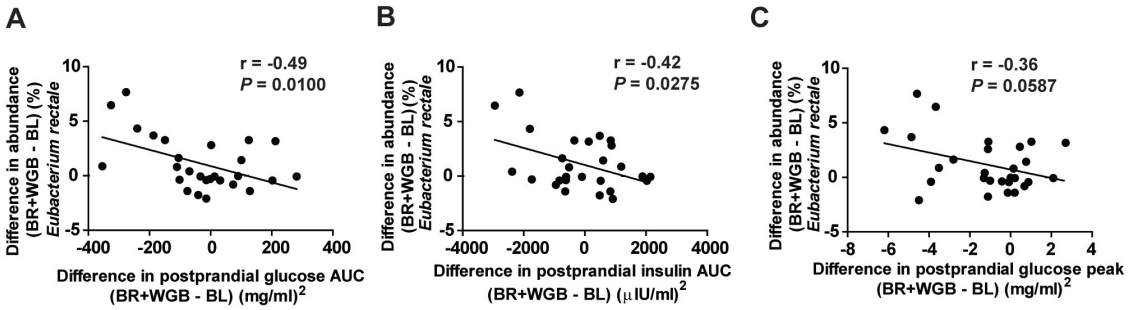

Figure 3.8: **Association between diet induced shifts in glycemic response and the proportion of *Eubacterium rectale*.** Correlation of the shift of the *Eubacterium rectale* abundance with the shifts observed in postprandial AUC (A), Insulin AUC (B), and maximum glucose levels (C). Shift refers to differences between values obtained during the BR+B period and the baseline. Pearsons r correlation and the P values are presented.

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Chapter 4

Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota

Inés Martínez*, Grant Wallace*, Chaomei Zhang, Ryan Legge, Andrew K. Benson, Timothy P. Carr, Etsuko N. Moriyama and Jens Walter. *Appl. Environ. Microbiol.*, 75:4175–4184, 2009.

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4.1 Abstract

The mammalian gastrointestinal microbiota exerts a strong influence on host lipid and cholesterol metabolism. In this study, we have characterized the interplay among diet, gut microbial ecology, and cholesterol metabolism in a hamster model of hypercholesterolemia. Previous work in this model had shown that grain sorghum lipid extract (GSL) included in the diet significantly improved the high-density lipoprotein (HDL)/non-HDL cholesterol equilibrium (T. P. Carr, C. L. Weller, V. L. Schlegel, S. L. Cuppett, D. M. Guderian, Jr., and K. R. Johnson, J. Nutr. 135:2236-2240, 2005). Molecular analysis of the hamsters' fecal bacterial populations by pyrosequencing of 16S rRNA tags, PCR-denaturing gradient gel electrophoresis, and *Bifidobacterium*-specific quantitative real-time PCR revealed that the improvements in cholesterol homeostasis induced through feeding the hamsters GSL were strongly associated with alterations of the gut microbiota. Bifidobacteria, which significantly increased in abundance in hamsters fed GSL, showed a strong positive association with HDL plasma cholesterol levels ($r=0.75$; $p=0.001$). The proportion of members of the family Coriobacteriaceae decreased when the hamsters were fed GSL and showed a high positive association with non-HDL plasma cholesterol levels ($r=0.84$; $p=0.0002$). These correlations were more significant than those between daily GSL intake and animal metabolic markers, implying that the dietary effects on host cholesterol metabolism are conferred, at least in part, through an effect on the gut microbiota. This study provides evidence that modulation of the gut microbiota-host metabolic interrelationship by dietary intervention has the potential to improve mammalian cholesterol homeostasis, which has relevance for cardiovascular health.

4.2 Introduction

The mammalian gut microbiota interacts intimately with its host, affecting both host metabolic and immunological phenotypes with important consequences for health (Gordon and Pesti, 1971, Hooper and Gordon, 2001, Nicholson et al., 2005). Recent studies have revealed complex linkages between the gut microbiome and host metabolism, with the microbes exerting effects on the energy balance by influencing glucose and lipid metabolism (Bäckhed et al., 2004a, Cani et al., 2007, Martin et al., 2007). This intimate metabolic relationship is most likely the consequence of a long coevolutionary process that resulted in a mutualistic relationship between the host and its microbial partners (Ley et al., 2006). However, life in industrialized societies has introduced profound changes into the human environment (e.g., diet, antibiotics, hospital deliveries, hygiene, etc.) that are markedly different from the conditions to which humans have evolved and that are likely to have occurred too abruptly for the human microbiome to adjust. Consequently, aberrations of the gut microbiota induced through lifestyle factors could be relevant to the etiology of several complex human diseases whose occurrence has markedly increased in developed countries. Interestingly, imbalances in the gut microbiota have been reported for obesity, type 1 and 2 diabetes, some allergies, and inflammatory bowel diseases in humans and animal models (Cani et al., 2007, Ley et al., 2005, Tannock, 2008, Turnbaugh et al., 2006, Wen et al., 2008). The connection between gut bacteria and disease suggests an intriguing paradigm on how to view and potentially treat complex diseases. Specific bacterial populations in the intestine could be pharmaceutical targets to maintain or restore metabolic functions (Cani and Delzenne, 2007, Flint et al., 2007).

Coronary heart disease (CHD) continues to be a major cause of death in developed countries and is another example of a western disease that is less common in un-

derdeveloped countries but increases in frequency with adoption of western customs (Bickler and DeMaio, 2008). Most risk factors for CHD (obesity, high blood pressure, type 2 diabetes, heredity, high cholesterol , and diet) have been linked to the gut microbiota (Cani et al., 2007, Flint et al., 2007, Holmes et al., 2008, Midtvedt, 1999, Turnbaugh et al., 2008), and gut bacteria have been suggested to play a role in the etiology of cardiovascular disease (Fava et al., 2006, Ordovas and Mooser, 2006). Cholesterol metabolism is a key factor in susceptibility to CHD, and as early as 1959, it has been shown that germfree rats have higher serum cholesterol concentrations than their conventional counterparts do (Danielsson and Gustafsson, 1959). Several mechanisms have been proposed by which gut bacteria could influence host cholesterol metabolism (Delzenne et al., 2006). Bacterial conversions of bile acids (such as the formation of secondary bile acids) are likely to play a role, as they affect enterohepatic circulation, de novo synthesis of bile acids, emulsification, and cholesterol absorption (Cowles et al., 2002, Martin et al., 2007, Midtvedt, 1999). A further mechanism by which gut bacteria might influence cholesterol metabolism is through Fiaf (fasting-induced adipocyte factor), which is selectively suppressed in the intestinal epithelium by the gut microbiota (Bäckhed et al., 2004a,b). Fiaf is an important regulator of lipid metabolism (e.g., through its inhibition of lipoprotein lipase) and has been shown to increase total cholesterol and high-density lipoprotein (HDL) cholesterol levels when overexpressed in transgenic mice (Mandard et al., 2006).

There are several reasons why hamsters are an excellent model for studying the metabolic relationships among diet, cholesterol metabolism, and gut microbiota in relation to health. First, hamsters are omnivorous, and their blood lipid profiles respond to diets in a predictive manner similar to humans (Bravo et al., 1994). Second, unlike mice and rats which lack cholesterol ester transfer protein, hamsters exhibit all of the enzymatic pathways in lipoprotein and bile metabolism that are also present

in humans. They exhibit limited hepatic synthesis of cholesterol and bile acids, resulting in more relevant data when extrapolating to humans (Horton et al., 1995). Third, hamsters develop atherosclerosis in a predictive manner in response to dietary manipulation (Mitchell and McLeod, 2008).

Using the Golden Syrian hamster model, Carr and coworkers have shown that the hexane-extractable lipid fraction of grain sorghum whole kernels (GSL), when included in the hamsters' diet, leads to a significant reduction of plasma non-HDL and liver cholesterol levels while increasing HDL cholesterol levels (Carr et al., 2005). We extended this researc h and performed a comprehensive molecular characterization of the fecal microbiota of the hamsters by pyrosequencing of 16S rRNA tags, denaturing gradient gel electrophoresis (DGGE), and Bifidobacterium specific quantitative real-time PCR (qRT-PCR) in order to test whether metabolic effects of GSL were associated with specific modifications of the gut microbiota.

4.3 Materials and methods

4.3.1 Animal experiments

The fecal samples analyzed here were obtained during a previous study that determined the effect of GSL included in the diet on the cholesterol metabolism of hamsters, and the handling of animals, feed composition, GSL composition, sample collection, and preparation have been described previously (Carr et al., 2005). Briefly, groups of seven or eight male F1B Syrian hamsters (Bio Breeders, Watertown, MA) were housed in cages (each hamster in an individual cage) and kept at 25C with a 12-h light-12-h dark cycle. Hamsters were fed a modified AIN-93 M diet (Reeves et al., 1993) supplemented with 0%, 1% and 5% grain sorghum lipid extract at the expense

of cornstarch. Daily feed intake was determined to assess individual GSL ingestions. GSL was prepared from whole kernels obtained from a mixture of commercial red grain sorghum hybrids grown in Nebraska. Hamsters had free access to food and water throughout the study. After the hamsters were on their respective diets for 3 weeks, the complete fecal output for each hamster was collected over 7 days. The fecal samples were ground, weighed, and stored frozen at -80°C until the DNA extractions were performed.

4.3.2 DNA extraction

The fecal samples were diluted in ice-cold phosphate-buffered saline (pH 7) in a 1:10 ratio and centrifuged at 8,000 xg for 5 min. This washing step was repeated two times. Bacterial cell pellets were resuspended in 750 μl lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, 20 mg/ml lysozyme) and transferred to a microcentrifuge tube containing 300 mg of 0.1-mm zirconium beads (BioSpec Products). Samples were then incubated at 37°C for 20 min followed by the addition of 85 μl of 10% sodium dodecyl sulfate solution and 40 μl proteinase K (15 mg/ml). After incubation for 15 min at 60°C, 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the samples were homogenized in a MiniBeadbeater-8 (BioSpec Products) at maximum speed for 2 min. Samples were cooled on ice before the layers were separated by centrifugation at 10,000 xg for 5 min. The top layer was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol; DNA was recovered by standard ethanol precipitation. The DNA pellets were dried for 30 min at room temperature and later resuspended in 100 μl of Tris-HCl buffer (10 mM, pH 8.0).

4.3.3 Analysis of the gut microbiota of hamsters by pyrosequencing of 16S rRNA tags

The V1-V3 region of the 16S rRNA gene was amplified by PCR using bar-coded universal primers 8F and 518R containing the A and B sequencing adaptors (454 Life Sciences). The forward primer (A-8FM) was 5-gcctccctcgcgccatcagAGAGTTTGATCMTGGCTCAG-3 where the sequence of the A adaptor is shown in lowercase letters. The reverse primer (B-518) was 5-gccttgccagcccgctcagNNNNNNNNATTACCGCGGCTGCTGG-3 where the sequence of the B adaptor is shown in lowercase letters and N represents an eight-base bar code that is unique for each sample. Prior to sequencing, amplicons from the individual PCR samples were quantified using the Quant-iT PicoGreen double-stranded DNA assay (Invitrogen) and quality controlled on an Agilent 2100 bioanalyzer. The amplicons from each reaction mixture were mixed in equal amounts based on concentration and subjected to emulsion PCR, and amplicon libraries were generated as recommended by 454 Life Sciences. Sequencing was performed from the B end using the 454/Roche B sequencing primer kit using a Roche Genome Sequencer GS-FLX using the standard protocol. Samples were combined in a single region of the picotiter plate such that approximately 1,000 to 2,000 sequences were obtained from each animal. The data analysis pipeline removed low-quality sequences (i) that do not perfectly match the PCR primer at the beginning of a read, (ii) that are shorter than 200 bp in length, (iii) that contain more than two undetermined nucleotides (N), or (iv) that do not match a bar code. Sequences (1,000 to 2,000 per animal) were quality controlled and binned according to bar codes.

Taxonomy-based analyses were performed by assigning taxonomic status to each sequence using the CLASSIFIER program of the Ribosomal Database Project (Wang et al., 2007). To estimate species richness and diversity, taxonomy-independent meth-

ods wer used. Sequences were aligned using Infernal Aligner; sequences from individual animals and then pooled sequences from all animals of a single treatment group were aligned. Cluster analysis was performed using the complete linkage clustering algorithm available through the Pyrosequencing pipeline of the Ribosomal Database Project (Cole et al., 2009). Clustering was done with a 97% cutoff for inclusion into an operational taxonomic unit (OTU) and was performed on alignments of sequences from individual animals. The number of species and species richness were estimated by further sampling-based (rarefaction) analyses of OTU data and of calculated Shannon diversity indices.

4.3.4 PCR-DGGE analysis

PCR was performed using universal primers PRBA338fGC (5-CGCCCGCCGCGCG-CGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3) - and PRUN518r (5-ATTACCGCGGCTGCTGG-3 (Ovreas et al., 1997), which amplify the V3 region of the 16S rRNA gene. DGGE was performed by the method of Walter and coworkers (Walter et al., 2000) using a DCode universal mutation detection system (Bio-Rad, Hercules, CA). DNA bands in the DGGE gel were visualized by standard ethidium bromide staining and photographed using the InGenius gel documentation system (Syngene, Frederick, MD). DGGE images were analyzed using BioNumerics software version 5.0 (Applied Maths, Kortrijk, Belgium). Bands were manually assigned, and the normalized banding patterns were used to generate distance matrices by calculating the Pearson product moment correlation coefficients for all pair-wise combinations of patterns. This approach compares profiles in a pair-wise manner based on the entire densitometric curve, therefore accounting for both band position and intensity. DGGE fingerprints were transformed to peak profiles using

the BioNumerics software, and the intensities of individual bands were determined as a percentage of the peak surface area relative to the surface area of the entire molecular fingerprint of the sample. To determine the effects of feeding hamsters GSL, normalized fragment intensities of all bands in DGGE fingerprints were determined and compared for the feeding groups.

In order to identify species represented by bands detected by DGGE, bands of fecal fingerprints from two or three animals were excised from the gel, purified, and reamplified by the method of ben Omar and Ampe (ben Omar and Ampe, 2000), and cloned using the TOPO TA Cloning kit for sequencing (Invitrogen). Plasmids were isolated from three transformants per band using the QIAprep spin minprep kit (Qiagen), and inserts were sequenced by a commercial provider following the manual of the cloning kit. Closest relatives of the partial 16S rRNA sequences were determined using the nucleotide blast web tool at the NCBI website (website) and the Seqmatch web tool provided through the Ribosomal Database Project (website). A phylogenetic tree was generated from the consensus sequence of the F bands in three individual animals using the unweighted-pair group method using average linkages and neighbor-joining algorithms in the MEGA4 software package (Tamura et al., 2007). There were a total of 178 positions in the final data set, and the evolutionary distances were computed by using the Kimura two-parameter method and are reported as the number of base substitutions per site.

4.3.5 Specific quantification of bifidobacteria by qRT-PCR

Quantification of total bifidobacteria was performed by quantitative real-time PCR using primers BifFor (5-TCGCGTCYGGTGTGAAAG-3) and BifRev (5-CCACATCCAGCRTCCAC-3) (Rinttila et al., 2004). PCRs were performed using a Master-

cycler Realplex2 (Eppendorf AG, Hamburg, Germany). Each PCR was done in a 25 μl volume. The reaction mixture comprised 11.25 μl of the 20 SYBR solution and 2.5 RealMasterMix (Eppendorf AG, Hamburg, Germany), 0.5 μM of each primer, and 1 μl of DNA template. The amplification program consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles, where 1 cycle consisted of 15 s at 95°C (denaturation), 20 s at 58°C (annealing), and 30 s at 68°C (extension), and fluorescence at each step was measured. To control the specificity of the amplifications, a melting curve was done consisting of a denaturation step of 15 s at 95°C, an increase from 58°C to 95°C over a 20 min period, and a final step of 15 s at 95°C. Cultures of *B. animalis* ATCC 25527^{T} and *B. infantis* ATCC 15697^{T} were used to generate standard curves for absolute quantification of bifidobacteria in the fecal samples. Bacterial counts of overnight cultures (12 h) were determined by plate counting, and a 10-fold dilution series was performed in phosphate-buffered saline buffer for each strain. DNA was isolated from individual samples of the dilution series using the method for fecal samples. Standard curves were made by plotting the threshold cycle values obtained from DNA of the dilution series as a linear function of the base 10 logarithm of the number of bifidobacteria. Two individual qRT-PCR runs of all fecal DNA templates in duplicate were performed, and means of all four values were used for the analysis. Despite the use of two different strains of bifidobacteria to generate one standard curve, its correlation coefficient r^2 was >0.96.

To quantify the *Bifidobacterium animalis*-like phylotype detected by DGGE, we used a specific primer (Bh1) based on a highly variable region of the sequence of fragment F in the DGGE gel (5-GGCAGGGGGTTTTCCTC-3). This primer was used in combination with primer BifRev (Rinttila et al., 2004) used for the *Bifidobacterium* genus-specific qRT-PCR. PCR was performed as described above. The specificity of the PCR was tested using DNA isolated from fecal samples from 10 human sub-

jects and DNA from *B. animalis* ATCC 25527^{T} and *Bifidobacterium infantis* ATCC 15697^{T}. The PCRs all gave negative results with the primer combination Bh1 and BifRev and positive results with primers BifFor and BifRev (data not shown). As we had no cultural representative of the phylotype represented by band F, we used a standard curve generated as described above with *B. animalis* ATCC 25527^{T} and *B. infantis* ATCC 15697^{T} and primers BifFor and BifRev. Although the standard curve was generated with a different forward primer, it can be assumed that no significant bias is introduced, as the efficiencies of the two PCR systems were virtually identical (0.51 for primers BifFor and BifRev and 0.56 for primers Bh1 and BifRev).

4.3.6 Correlation analysis of gut microbiota-host metabolic functional relationships

Correlation analysis between metabolic host parameters and bacterial populations at different taxonomic levels was performed by the method of Cani and coworkers (Cani et al., 2007). Metabolic parameters included in the correlation analysis were the levels of cholesterol absorption, fecal cholesterol, plasma total cholesterol, plasma HDL cholesterol, plasma non-HDL cholesterol, liver total cholesterol, liver-free cholesterol, liver triglycerides, liver phospholipids, and liver-esterified cholesterol. The determination of these metabolic phenotypes and the methods applied were reported previously (Carr et al., 2005).

4.3.7 Genome comparisons

The web-based Integrated Genomics Platform of the Joint Genome Institute (JGI) was used to identify functions enriched in bifidobacteria (Markowitz et al., 2007). The Abundance Profile Search was used to identify clusters of orthologous groups

of proteins (COGs) that were more abundant in individual bifidobacterial genomes (*Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium adolescentis* L2-32, *Bifidobacterium animalis* subsp. *lactis* HN019, *Bifidobacterium dentium* ATCC 27678, *Bifidobacterium longum* DJO10A, and *Bifidobacterium longum* NCC2705) compared to the genomes of a selection of bacteria commonly present in the mammalian gastrointestinal tract (*Bacteroides caccae* ATCC 43185, *Bacteroides capillosus* ATCC 29799, *Bacteroides fragilis* NCTC 9343, *Bacteroides fragilis* YCH46, *Bacteroides ovatus* ATCC 8483, *Bacteroides stercoris* ATCC 43183, *Bacteroides thetaiotaomicron* VPI-5482, *Bacteroides uniformis* ATCC 8492, *Bacteroides vulgatus* ATCC 8482, *Clostridium acetobutylicum* ATCC 824, *Clostridium bartlettii* DSM 16795, *Clostridium bolteae* ATCC BAA-613, *Clostridium leptum* DSM 753, *Clostridium ramosum* DSM 1402, *Clostridium* sp. strain L2-50, *Clostridium* sp. strain SS2/1, *Clostridium thermocellum* ATCC 27405, *Collinsella aerofaciens* ATCC 25986, *Coprococcus eutactus* ATCC 27759, *Dorea formicigenerans* ATCC 27755, *Dorea longicatena* DSM 13814, *Enterobacter* sp. strain 638, *Enterococcus faecalis* V583, *Enterococcus faecium* DO, *Escherichia coli* K-12, *Eubacterium dolichum* DSM 3991, *Eubacterium siraeum* DSM 15702, *Eubacterium ventriosum* ATCC 27560, *Faecalibacterium prausnitzii* M21/2, *Lactobacillus reuteri* 100-23, *Lactobacillus reuteri* F275, *Lactobacillus salivarius* subsp. *salivarius* UCC118, *Methanobrevibacter smithii* ATCC 35061, *Parabacteroides distasonis* ATCC 8503, *Parabacteroides merdae* ATCC 43184, *Peptostreptococcus micros* ATCC 33270, *Providencia stuartii* ATCC 25827, *Ruminococcus gnavus* ATCC 29149, *Ruminococcus obeum* ATCC 29174, *Ruminococcus torques* ATCC 27756, and *Salmonella enterica* serovar Typhimurium LT2). Functions associated with lipid metabolism were specifically selected from the enriched COGs and added to the function list. A function profile of these COGs was then generated for all of the gut species. Please refer to the IMG web page for details (website).

4.3.8 Statistical analysis

Results are presented as means standard deviations (SDs). Statistical tests for treatment effects of the GSL on the abundance of individual taxonomic ranks or DGGE band intensities were performed by one-way analysis of variance (ANOVA) analysis followed by Tukey's posthoc multiple comparison tests. The Mann-Whitney test was used to compare Shannon diversity indices of gut populations. Correlations between metabolic parameters and bacterial populations were assessed by Pearson's correlation test using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA).

4.4 Results

4.4.1 Characterization of the hamster gut microbiota by pyrosequencing of 16S rRNA tags

To determine whether proportional changes of the gut microbiota were associated with the effects of GSL on cholesterol metabolism in hamsters, we analyzed the fecal microbiota of hamsters fed 0% (n = 7), 1% (n = 7), and 5% (n = 7) GSL by pyrosequencing of the V3 region of the 16S rRNA gene. A total of 34,424 sequences were studied; the average sequence length was around 250 bp, and an average of 1,639 sequences per animal were studied. Taxonomy-based analysis showed that the composition of the hamster gut microbiota at the phylum level is similar to that of humans and mice, being dominated by Firmicutes and Bacteroidetes. An unusual feature of the hamster gut microbiota, however, was that Firmicutes comprised the vast majority of the taxa (94%) with Bacteroidetes making up only 4% of the population. The remaining bacteria belonged to the phyla Verrucomicrobia and Actinobacteria (each representing around 1% of the total sequence tags) and Proteobacteria and candidate

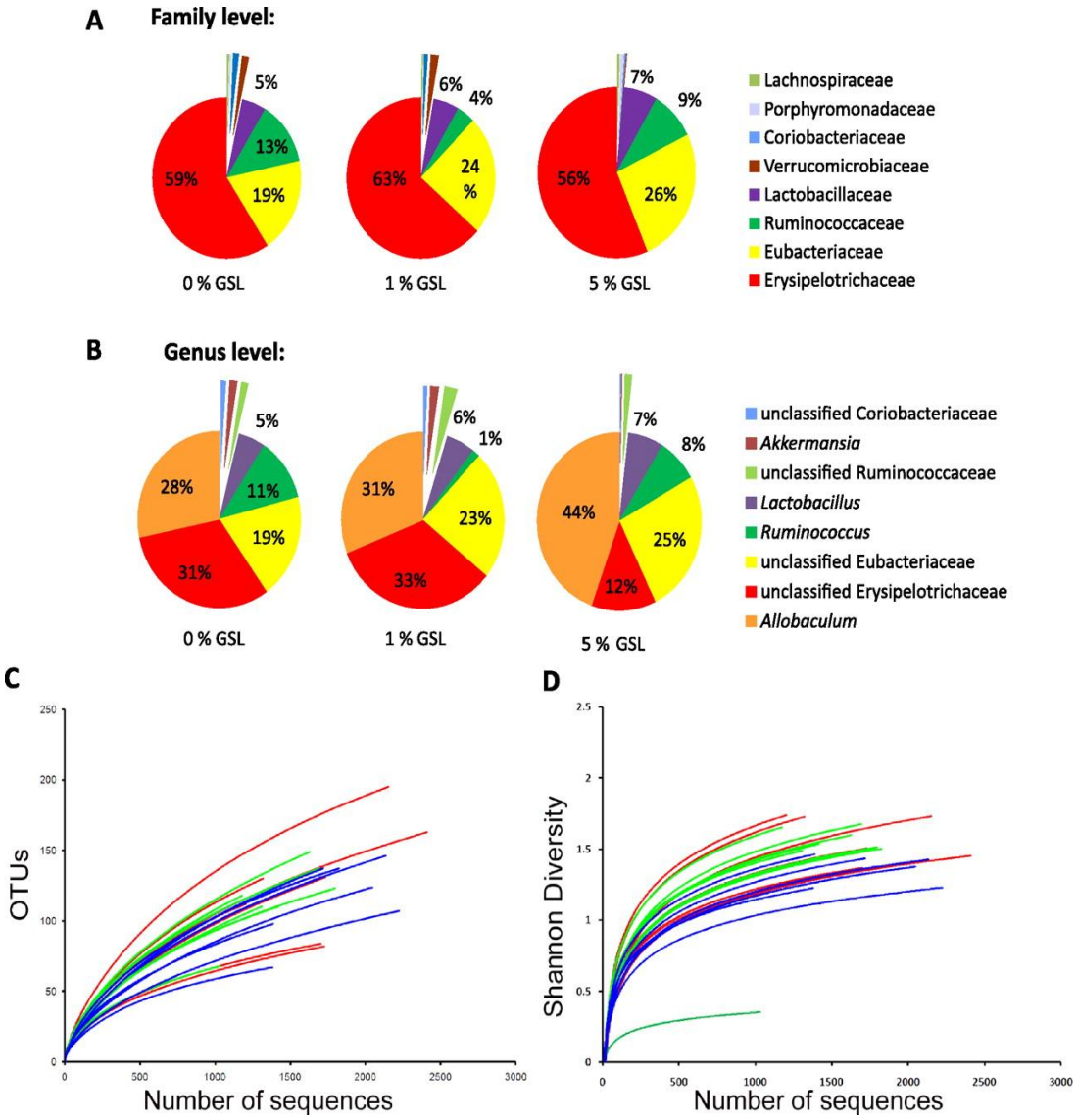

Figure 4.1: **Characterization of the gut microbiota composition of hamsters fed different amounts of GSL as determined by pyrosequencing of 16S rRNA tags (V3 region)**. Composition of the gut microbiota of hamsters fed 0%, 1%, and 5% GSL (n = 7 per group) at the family level (A) and the genus level (B). (C) Rarefaction curves of OTUs from sequences of fecal samples from individual hamsters fed 0% GSL (red), 1% GSL (green), and 5% GSL (blue). (D) Shannon diversity indices of the gut microbiota of individual hamsters fed 0% GSL (red), 1% GSL (green), and 5% GSL (blue). OTUs were identified using 97% cutoffs for rarefaction and Shannon diversity indices.

division TM7 (0.07% and 0.024% of sequences, respectively).

At the family level, the predominant groups in hamsters of the control group were the Erysipelotrichaceae, Eubacteriaceae , Ruminococcaceae, and Lactobacillaceae, represented by an average of 59%, 19%, 13% and 5% of the total fecal microbiota, respectively (Figure 4.1A). Of the bacterial groups on the genus level, the most dominant were unclassified Erysipelotrichaceae, *Allobaculum*, unclassified Eubacteriaceae, *Ruminococcus*, and *Lactobacillus*, comprising 31%, 28%, 19%, 11%, and 5% of the total sequence pool on average in control animals, respectively (Figure 4.1B). With the exception of *Allobaculum*, these genera are also shared with the gut microbiota reported for mice, humans, and primates (Eckburg et al., 2005, Ley et al., 2005, McKenna et al., 2008, Turnbaugh et al., 2008). As shown in Supplementary Figure 4.5 in the supplemental material, pyrosequencing revealed high animal-to-animal variability on both the family and genus levels.

4.4.2 Effects of GSL on specific taxa of the hamster gut microbiota

Sequence proportions determined by pyrosequencing were used to establish the effects of the GSL on the gut microbiota composition. To identify specific taxa that were affected by the feeding treatments, the proportions of taxa in each rank of each animal were tested for treatment effects. As shown in Table 4.1, ANOVA identified one family, the Coriobacteriaceae (p=0.042), and two bacterial groups at the genus level, unclassified members of the family Erysipelotrichaceae (p=0.0016) and genus *Pseudoramibacter* (p=0.017), as being significantly affected by the inclusion of GSL to the hamsters' diet. Moreover, values for the genus *Allobaculum* (p=0.096) and unclassified members of the family Coriobacteriaceae (p=0.064) approached statistical

Table 4.1: Abundance of bacterial groups in the fecal microbiota of hamsters that changed by including GSL in the diet as determined by pyrosequencing of 16S rRNA tags[a]

Bacterial taxa	Abundance of bacterial group[b](% of total sequences obtained with sample [mean ± SD]) in hamsters fed:		
	0% GSL	1% GSL	5% GSL
Family level			
Coriobacteriaceae	1.22 ± 0.79	0.79 ± 0.63	**0.31 ± 0.33**[c]
Genus level			
Allobaculum	27.82 ± 15.9	30.63 ± 15.8	**43.55 ± 7.0**[e]
Pseudoramibacter	0.11 ± 0.09	**0.48 ± 0.30**[c]	**0.35 ± 0.21**[c]
Unclassified members of the following families			
Coriobacteriaceae	1.0 ± 0.7	0.69 ± 0.62	**0.23 ± 0.31**[e]
Erysipelotrichaceae	31.0 ± 7.4	32.7 ± 14.0	**12.39 ± 6.0**[d]

[a] There were seven hamsters in each group.
[b] Values that were significantly different or approaching statistical significance are shown in boldface type.
[c] Statistically significantly different from the value for hamsters fed 0% GSL ($p<0.05$) by ANOVA.
[d] This value was statistically significantly different from the value for hamsters fed 0% GSL ($p<0.01$) and from the value for hamsters fed 1% GSL ($p<0.01$) by ANOVA.
[e] Approaching statistical significance compared to the value for hamsters fed 0% GSL ($p<0.1$) by ANOVA.

significance.

Taxonomy-independent analysis of the hamster gut microbiota from individual animals showed that with a conservative level of 97% identity as a cutoff for OTUs, nearly 200 OTUs were observed in the average of 1,600 sequences from each animal (Figure 4.1C). Individual animals in the 5% GSL feeding group showed a trend toward fewer OTUs in the rarefaction curves. The Shannon diversity indices from individual animals also showed a trend of fewer OTUs in animals fed 5% GSL (blue lines) compared to animals fed 0% GSL (Figure 4.1D). Grouping of the samples by GSL showed significant differences between 0% and 5% GSL ($p<0.0001$, Mann-Whitney

test). Thus, 5% GSL had the effect of reducing the diversity of the gut microbiota.

4.4.3 DGGE analysis of fecal microbiota of hamsters fed GSL

To validate the findings obtained with pyrosequencing, fecal bacterial populations of the hamsters were also analyzed by PCR-DGGE. The DGGE gel is shown in Figure 4.2A, and the results of analysis of the gel are presented in Table 4.2. Feeding the hamsters 5% GSL significantly increased the staining intensity of band C (p=0.037) and band F (p=0.011). Band A showed a high animal-to-animal variation, and no consistent impact of GSL was detected. Sequence analysis of amplicons from these bands revealed that they represent bacteria related to *Ruminococcus bromii* (band A), *Allobaculum stercoricanis* (band C), and *Bifidobacterium animalis* (band F). Phylogenetic comparison of the sequence from band F with the closest hits in the RDP database revealed this sequence to be most similar to Bifidobacterium animalis, and it is referred to as the *Bifidobacterium animalis*-like phylotype in this article (a phylogenetic tree is shown in Figure 4.2B).

Although providing less depth, DGGE analysis showed good agreement with the results from pyrosequencing. Both methods detected the increase of bacteria related to *Allobaculum* and the high animal-to-animal variability of bacteria related to *Ruminococcus*. Relative species quantification obtained with pyrosequencing showed high correlations with staining intensities of DGGE bands representing the same bacterial groups (for *Ruminococcus*, r=0.94; for *Allobaculum*, r=0.81; both p<0.0001) (see Supplementary Figure 4.6). This is remarkable, as both methods are only semi-quantitative and entail multitemplate PCR susceptible to PCR bias. The main difference between the findings by pyrosequencing and DGGE was in the proportions

Table 4.2: Ratio of staining intensities of major bands as a proportion of total fingerprint intensity and results of sequence analysis of selected bands

Band	Mean band intensity[a](SD) in DNA from hamsters fed:			Clostest Genebank hit[d]	Closest type strain[e]
	0% GSL	1% GSL	5% GSL		
A	20.0 (17.5)	**0.06 (0.16)**[b]	14.2 (14.3)	AM265444, uncultured bacteria, clone ratBD050202C (99.4)	*Ruminococcus bromii* ATCC 27255^T [L76600] (96.8)
B	5.6 (4.5)	7.8 (8.1)	6.2 (6.9)	ND[f]	ND
C	9.2 (7.9)	7.9 (4.3)	**17.4 (8.2)**[c]	EU777003, uncultured bacterium clone molerat_aai70g11 (92.4-93.0)	*Allobaculum stercoricanis* DSM 13633^T [AJ417075] (92.0-92.6)
D	11.4 (11.0)	13.1 (7.1)	10.3 (7.6)	ND	ND
E	3.4 (3.2)	7.5 (10.3)	4.3 (5.1)	ND	ND
F	5.7 (1.0)	6.2 (3.4)	**11.1 (3.7)**[b,c]	AB186296, *Bifidobacterium animalis* strain DBF 1307 (96.8)	*Bifidobacterium animalis* subsp. *animalis* DSM 10140^T [X89513] (96.8)
G	4.0 (1.6)	3.2 (2.2)	3.2 (1.3)	ND	ND
H	3.0 (2.2)	8.0 (7.6)	1.7 (2.0)	ND	ND

[a] Ratio of staining intensities of major bands as a proportion of total fingerprint intensity (shown as a percentage). Values that were significantly different are shown in boldface type.

[b] Statistically significantly different from the value for hamsters fed 0% GSL ($p<0.05$) by ANOVA.

[c] Statistically significantly different from the value for hamsters fed 1% GSL ($p<0.05$) by ANOVA.

[d] The GenBank accession number and species or clone is shown. The values in parentheses are the percentages of similarity.

[e] The closest type strain is shown first. The GenBank accession number is shown in brackets. The values in parentheses are the percentages of similarity.

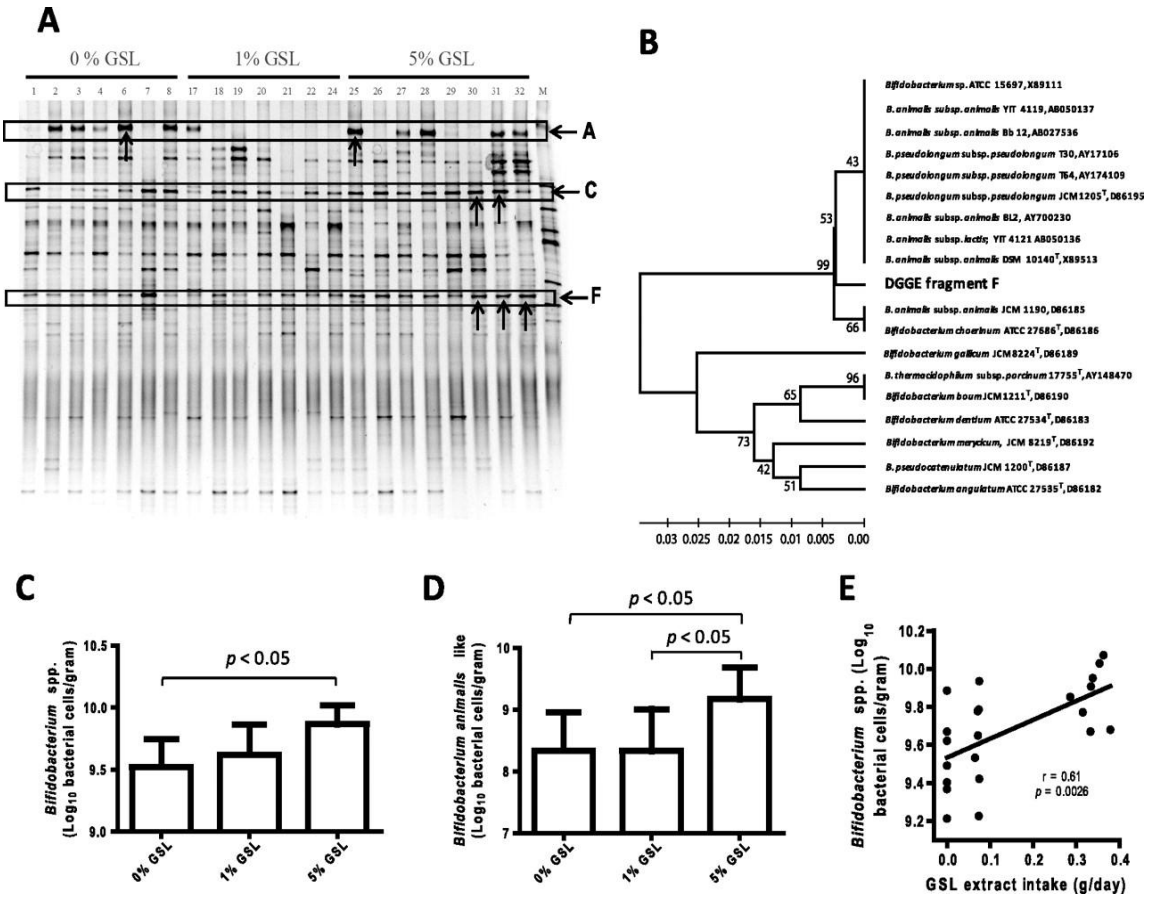

Figure 4.2: **Impact of GSL on the gut microbiota composition of hamsters fed 0% GSL (n = 7), 1% GSL (n = 7), and 5% GSL (n = 8) as determined by DGGE and qRT-PCR**. (A) DGGE showing fingerprints of DNA isolated from the fecal samples of hamsters. Lanes 1 to 32 contain DNA from individual hamsters. Lane M contains markers from reference strains. Bands C and F showed significant increases in staining intensity in fecal fingerprints of hamsters fed 5% GSL. The bands A, C, and F marked by an arrow were excised, purified, and sequenced (Table 4.2). (B) Phylogenetic tree of DGGE band F with sequences that revealed highest similarities in GenBank. The tree was inferred using the unweighted-pair group method using average linkages, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. A neighbor-joining tree resulted in essentially the same phylogeny (data not shown). (C) Cell numbers of total bifidobacteria in hamster fecal samples as determined by qRT-PCR. (D) Quantification of the *Bifidobacterium animalis* -like phenotype detected by DGGE in hamster fecal samples by qRT-PCR. (E) Correlation of cell numbers of bifidobacteria with daily GSL intake.

of bifidobacteria, where a significant proportion could be detected only by DGGE, while only 0.03% of the total sequences obtained by pyrosequencing corresponded to bifidobacteria. The difference can be explained by the use of primer 8F in pyrosequencing, which shows three mismatches with the 16S rRNA genes from five *Bifidobacterium* species for which whole-genome sequences were available (data not shown). Accordingly, many studies employing direct analysis of 16S rRNA genes to study the human gut microbiota and using the primer 8F, which is one of the most commonly used primers for such approaches, resulted in a significant underrepresentation of *Bifidobacterium* species (Eckburg et al., 2005, Palmer et al., 2007, Suau et al., 1999, Zoetendal et al., 2008).

4.4.4 Quantification of bifidobacteria using qRT-PCR

Since primer 8F resulted in an underrepresentation of bifidobacteria in pyrosequencing and to confirm and quantify the bifidogenic effect of the GSL detected by DGGE analysis, a specific qRT-PCR procedure was used to determine the numbers of total bifidobacteria and the *Bifidobacterium animalis*-like phylotype. As shown in Figures 4.2 C and D, qRT-PCR analysis showed a significant increase in cell numbers of total bifidobacteria ($p=0.012$) and the *Bifidobacterium animalis*-like phylotype ($p=0.019$). As shown in Figure 4.2E, the numbers of bifidobacteria from individual hamsters were highly variable, but a significant correlation between cell numbers and daily GSL intake was observed.

4.4.5 Bifidobacteria and Coriobacteriaceae showed high correlations with important markers of host cholesterol metabolism

In a previous study using the animals studied here, dietary GSL reduced cholesterol absorption, plasma non-HDL cholesterol concentrations, and liver esterified cholesterol levels, while raising plasma HDL cholesterol levels (Carr et al., 2005). To determine whether alterations of the gut microbiota in hamsters fed GSL were associated with an improvement in cholesterol metabolism, a correlation analysis was used to determine correlations between all bacterial taxa at different taxonomic levels and host metabolic phenotypes. The analysis revealed highly positive correlations between HDL plasma concentrations and total bifidobacteria (r=0.75, p=0.0011), between HDL plasma concentrations and *Bifidobacterium animalis*-like phylotype (r=0.77, p=0.0009), among total Coriobacteriaceae and non-HDL plasma concentrations (r=0.84, p=0.0002), and between unclassified Coriobacteriaceae and both non-HDL plasma concentration (r=0.82, p=0.0004) and cholesterol absorption (r=0.71, p=0.0042). These high correlations were observed only in animals fed 1% and 5% GSL, and inclusion of the values from control animals significantly reduced correlations (Table 4.3). Graphs showing the highest correlations between bacterial taxa and metabolic phenotypes are shown in Figure 4.3, and a metabolic network diagram linking GSL, bacterial phylotypes, and host cholesterol metabolism is shown in Figure 4.4. Interestingly, the correlations between bifidobacteria and HDL cholesterol concentration and between Coriobacteriaceae and non-HDL concentration showed higher significance than correlations achieved between GSL intake and the respective host metabolic phenotypes.

Table 4.3: Correlations between abundance of bacterial taxa and markers of cholesterol metabolism[a]

Bacterial taxa	Correlation[b](r value) between abundance of bacterial taxa and the following marker of cholesterol metabolism:			
	GSL intake	Non-HDL level	HDL level	Cholesterol absorption
Family level				
Coriobacteriaceae	-0.54 (-0.50)	**0.84** (0.37)	-0.56 (-0.18)	0.68 (0.50)
Genus level				
Bifidobacterium	0.56 (0.61)	-0.61 (-0.33)	**0.75** (0.34)	-0.42 (-0.51)
Allobaculum	0.50 (0.48)	-0.28 (-0.53)	0.24 (0.50)	-0.45 (-0.37)
Pseudoramibacter	-0.24 (0.23)	0.07 (-0.39)	-0.12 (0.19)	0.08 (-0.40)
Unclassified members of the following families				
Coriobacteriaceae	-0.48 (-0.52)	**0.82** (0.40)	0.52 (-0.19)	**0.71** (0.47)
Erysipelotrichaceae	**-0.74** (-0.70)	0.45 (0.34)	-0.48 (-0.32)	0.68 (0.56)

[a] Values for animals fed 1% and 5% GSL are presented.

[b] Values for all animals, including control animals, are presented in parentheses. Correlation coefficients with an r of <0.7 are shown in boldface type.

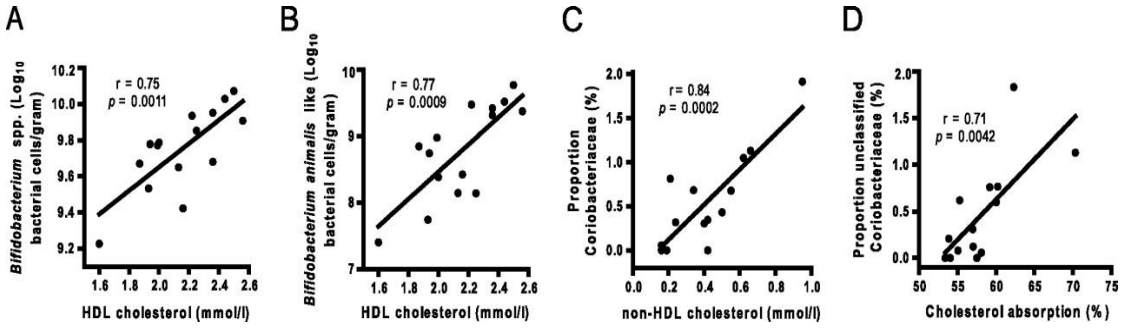

Figure 4.3: **Specific bacterial populations in the guts of hamsters show high associations with both cholesterol metabolic phenotypes and GSL intake**. (A and B) Correlations between cell numbers of total bifidobacteria (A) and the *Bifidobacterium animalis*-like phenotype (B) with HDL cholesterol. (C) Correlation between proportion of Coriobacteriaceae and non-HDL cholesterol. (D) Correlation between unclassified members of the family Coriobacteriaceae and cholesterol absorption. Data from control animals (0% GSL) were excluded from the analysis.

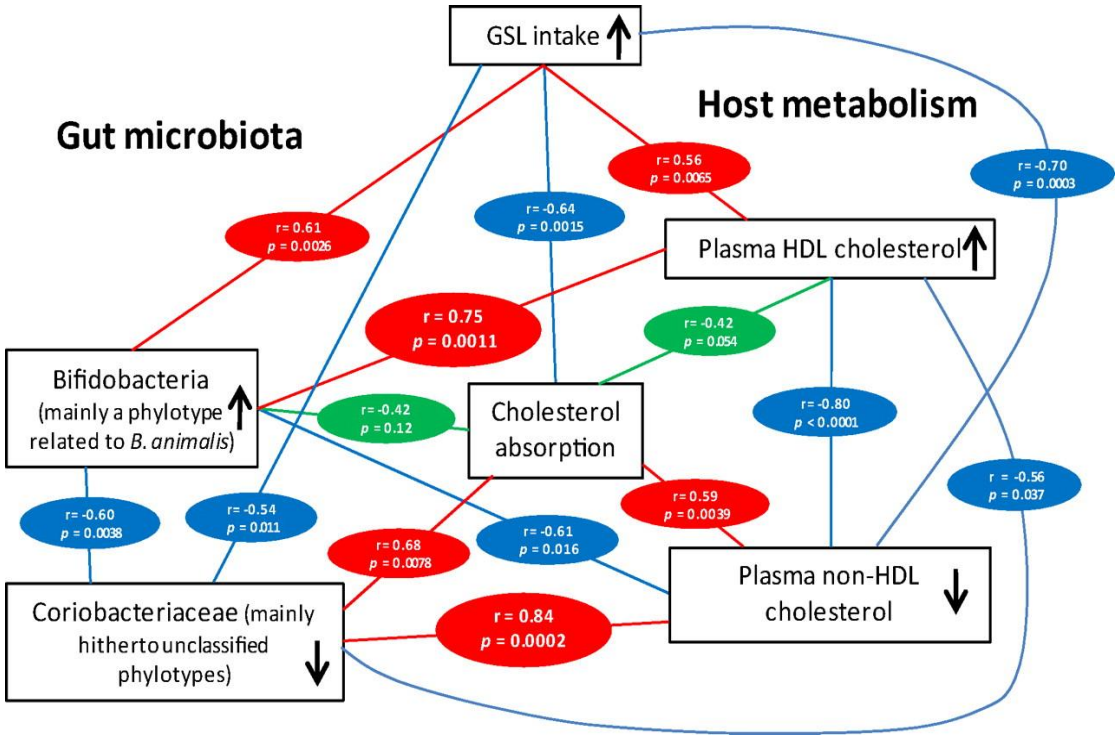

Figure 4.4: **Metabolic network showing the associations between daily GSL intake, gut microbiota composition, and host cholesterol metabolism in hamsters fed 0%, 1%, and 5% GSL**. Results of the correlations of cell numbers of bifidobacteria and proportions of Coriobacteriaceae and phenotypic markers were obtained with data from animals fed 1% and 5% GSL. Red connections indicate a positive correlation, while blue connections show correlations that are inverse. Green connections show associations with no statistical significance. Metabolic data were obtained by Carr and coworkers in a previous study (Carr et al., 2005).

4.4.6 Genome comparisons of bifidobacteria and other gut organisms

By comparing the relative abundance of different functional categories in 47 genomes of gut bacteria, we observed that proteins belonging to the COG clusters COG0400 (predicted esterase), COG0657 (esterase/lipase), and COG2272 (carboxylesterase type B) are enriched in six *Bifidobacterium* genomes (see Table 4.4 in the supplemental material). Carboxylesterases represented by COG0400 and COG2272 belong to enzymes that hydrolyze a wide variety of substrates, ranging from methylcaplyrate to p-nitrobenzyl (Hong et al., 1991, Zock et al., 1994).

4.5 Discussion

In humans, CHD is associated with high levels of low-density lipoprotein and low levels of high-density lipoprotein. The characterization of the gut microbiota in a hamster model of hypercholesterolemia showed that dietary intervention with GSL had a major impact on the composition of the gut microbiota and that these modulations were highly associated with improvements in the HDL and non-HDL cholesterol equilibrium. With consumption of GSL, population levels of bifidobacteria increased and showed a strong positive association with increases in HDL cholesterol levels. In contrast, relative abundance of members of the family Coriobacteriaceae decreased with feeding the hamsters GSL, and a high positive correlation with non-HDL cholesterol and cholesterol absorption was discovered. The findings indicate that GSL intake influences the HDL/non-HDL equilibrium, at least in part, through an alteration of the gut microbiota. We infer this because correlation coefficients between bifidobacterial and Coriobacteriaceae populations and plasma cholesterol concentrations were

higher than associations among GSL intake, host phenotypes, and cholesterol absorption (Figure 4.4). In addition, if bacterial phylotype/host phenotype correlations were merely a result of GSL affecting both bacterial taxa and cholesterol metabolism independently, one would assume that all bacterial taxa whose abundance correlated with GSL intake would show an association with host phenotypes. However, much lower correlation coefficients with non-HDL and HDL plasma concentrations were observed between relative abundance of unclassified members of the family Erysipelotrichaceae and the genus *Allobaculum*, although these taxa showed significant associations with GSL intake (Table 4.3). However, it should be considered that bifidobacteria and Coriobactericeae are just two of hundreds of groups, and other bacteria, independent of GSL administration, are likely to interact with host cholesterol metabolism.

Changes in the composition of the hamsters' gut microbiota induced by GSL consumption were limited to a relatively small number of bacterial groups (Tables 4.1 and 4.3). These compositional adjustments had the net effect of reducing the overall species richness (number of individual species per unit population). However, the overall composition of the microbiota at the phylum level was not affected by GSL. The reason for this finding was that increases of dominant bacterial taxa were 'balanced' by a reduction of related bacteria, leaving the relative proportions of higher taxonomic taxa unaffected. *Allobaculum* belongs to the family Erysipelotrichaceae, and uncharacterized bacteria of this family declined as *Allobaculum* increased with feeding the hamsters GSL. Thus, the overall proportions within the family Erysipelotrichaceae and the phylum Firmicutes were not changed. Similar findings were obtained for the phylum Actinobacteria, where numbers of bifidobacteria increased while the abundance of members of the family Coriobacteriaceae declined. As shown in Supplementary Figure 4.7, significant inverse correlations were obtained between these related bacterial groups in individual animals. Similar diet-induced compositional adjustments of

the gut microbiota that maintain the overall composition at higher taxonomic levels have also been observed in humans. For example, the decline of bacteria belonging to the *Roseburia* and *Eubacterium rectale* groups induced through reduced carbohydrate intake was balanced by an increase in the number of related members of the *Clostridium coccoides* cluster in human fecal samples (Duncan et al., 2008). Furthermore, the diet of human infants appears to influence the *Bifidobacterium*/Coriobacteriaceae ratio, with higher numbers of bifidobacteria in breast-fed infants while there were higher numbers of coriobacteria when the infants were fed formula (Harmsen et al., 2000). Collectively, these findings indicate that homeostatic reactions that restore the overall equilibrium of the gut microbiota are often a natural consequence of compositional changes induced through diet. Nevertheless, as indicated by the correlation analysis in our study, an alteration of the gut microbiota at lower taxonomic levels is still likely to have important functional consequences for the host.

The mechanisms by which bifidobacteria and coriobacteria affect cholesterol metabolism remain an important field of future research. Including GSL in the diet reduced cholesterol absorption efficiency, which was directly correlated with non-HDL cholesterol concentration (Carr et al., 2005). The high correlations of unclassified members of the family Coriobacteriaceae with both non-HDL cholesterol and cholesterol absorption suggest that these bacteria could have a negative impact on cholesterol homeostasis through increasing cholesterol absorption. Bifidobacteria, on the other hand, showed high positive correlation with HDL cholesterol levels and no association with cholesterol absorption. Bifidobacteria have been shown to affect cholesterol and lipid metabolism in animal models when administered as probiotics or when stimulated by prebiotics (Crittenden and Playne, 2006, Delzenne et al., 2006). The mechanism by which bifidobacteria achieve these effects remain speculative, but they might impact cholesterol metabolism indirectly by suppressing numbers of Coriobac-

teriaceae. For both bacterial groups, the capability to transform bile acids has been reported (Ridlon and Hylemon, 2006), and this phenotypic trait might influence host cholesterol metabolism through an impact on enterohepatic circulation. The strong correlations between bacterial taxa and cholesterol metabolism were observed only in animals fed GSL and not in control animals, suggesting that GSL influences the relative abundance of these organisms as well as metabolic characteristics.

The consumption of lipids has not yet been associated with increases in numbers of intestinal bifidobacteria. In contrast, Cani and coworkers (Cani et al., 2007) showed that a high-fat diet significantly lowered the number of bifidobacteria in mice. The composition of the GSL administered to the hamsters in our study contained not only mono-, di-, and triglycerides but also esters, alcohols, and other lipophilic compounds, such as waxes, sterols, and polycosanols (Carr et al., 2005). Carbohydrates or fiber are an unlikely explanation for the bifidogenic effect of GSL, as the amounts of fiber in hexane extracts of grains are negligible (Carr et al., 2005). Interestingly, genome comparisons revealed that bifidobacteria possess metabolic capacities that could allow them to utilize complex lipids, including lipids that may not be utilized by other members of the gut microbiota or the host. Schell and colleages detected four genes encoding long-chain fatty acyl-coenzyme A synthetases in the genome of *Bifidobacterium longum*, more than any other prokaryote genome available at that time, except for *Streptomyces coelicolor* and another gastrointestinal tract inhabitant, *Bacteroides fragilis* (Schell et al., 2002). These findings together with the enrichment of putative esterases in bifidobacterial genomes detected above indicate that bifidobacteria are likely to utilize specific components of GSL leading to the increase in numbers in the gut.

The correlation analysis identified the *Bifidobacterium*/Coriobacteriaceae equilibrium to be important for the plasma cholesterol levels in hamsters, with bifidobacteria

being beneficial and coriobacteria being detrimental. While extrapolation of our observations to humans is still speculative, our findings suggest that bifidobacteria and coriobacteria could be potential targets for the prevention of metabolic aberrations that play a role in CHD. Clearly, it will be essential to first identify the exact bacterial taxa within the human gut microbiota that have strong correlations to cholesterol metabolism, which in itself is a challenging task. Unlike the inbred population of hamsters used in our study, human subjects have significant genetic diversity, and genetic factors that affect cholesterol metabolism play a more important role in humans than in the animal model. Furthermore, human subjects follow individual lifestyles and consume different diets, and they harbor variable and individual communities of the gut bacteria. All these factors will hamper the identification of bacterial contributors to human cholesterol metabolism. Nevertheless, it is tempting to speculate that the positive impact of breast-feeding on the *Bifidobacterium*/Coriobacteriaceae ratio in human infants (Harmsen et al., 2000) could be responsible for the higher HDL cholesterol levels observed in adults that were breast-fed in infancy (Parikh et al., 2007).
This study provides new and important perspectives on dietary modulation of the mammalian gut microbiota and its effects on the host. The findings indicate that a complex mixture of lipids can exert a prebiotic effect that leads to improvements in host cholesterol metabolism. In conclusion, this study provided evidence that modulation of bacterial populations in the gut has the potential to improve mammalian cholesterol homeostasis, which has relevance in the prevention of CHD.

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4.7 Supplementary material

Table 4.4: COGs involved in lipid metabolism identified as enriched in the genomes of bifidobacteria.

Function ID	Name	Bif ado 153	Bif ado L22	Bif ani HN9	Bif den 278	Bif lon DJA	Bif lon NC5	Met smi 351	Col aer 256	Bac cac 435	Bac fra 933	Bac fra YC6	Bac ova 843	Bac ste 433	Bac the VP2	Bac uni 842	Bac vul 842
COG0400	Predicted esterase	1	1	2	2	3	3	0	0	0	0	0	0	0	0	0	0
COG0657	Esterase/lipase	2	4	2	3	6	4	0	3	1	2	3	3	1	5	3	2
COG2272	Carboxylesterase type B	2	4	1	0	0	0	0	0	0	0	0	0	0	0	1	0

Function ID	Name	Par dis 853	Par mer 434	Ent fae V53	Ent fae DOO103	Lac reu F25	Lac reu UC8	Lac sal 299	Bac cap 824	Clo ace 165	Clo bar BA3	Clo bol 753	Clo lep L20	Clo sp. SS1	Clo sp. 275	Clo the 275	Dor for
COG0400	Predicted esterase	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0
COG0657	Esterase/lipase	4	4	3	2	4	4	1	2	2	2	4	1	3	1	1	5
COG2272	Carboxylesterase type B	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

Function ID	Name	Dor lon 134	Fae pra M22	Eub sir 152	Cop eut 279	Rum gna 299	Rum obe 294	Rum tor 276	Pep mic 330	Eub dol 391	Eub ven 270	Clo ram 142	Ent sp. 638	Esc col K12	Sal typ LT2	Pro stu 257
COG0400	Predicted esterase	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
COG0657	Esterase/lipase	3	5	1	2	2	3	1	1	4	3	1	2	1	1	0
COG2272	Carboxylesterase type B	0	0	0	0	0	0	0	0	0	2	0	1	0	1	0

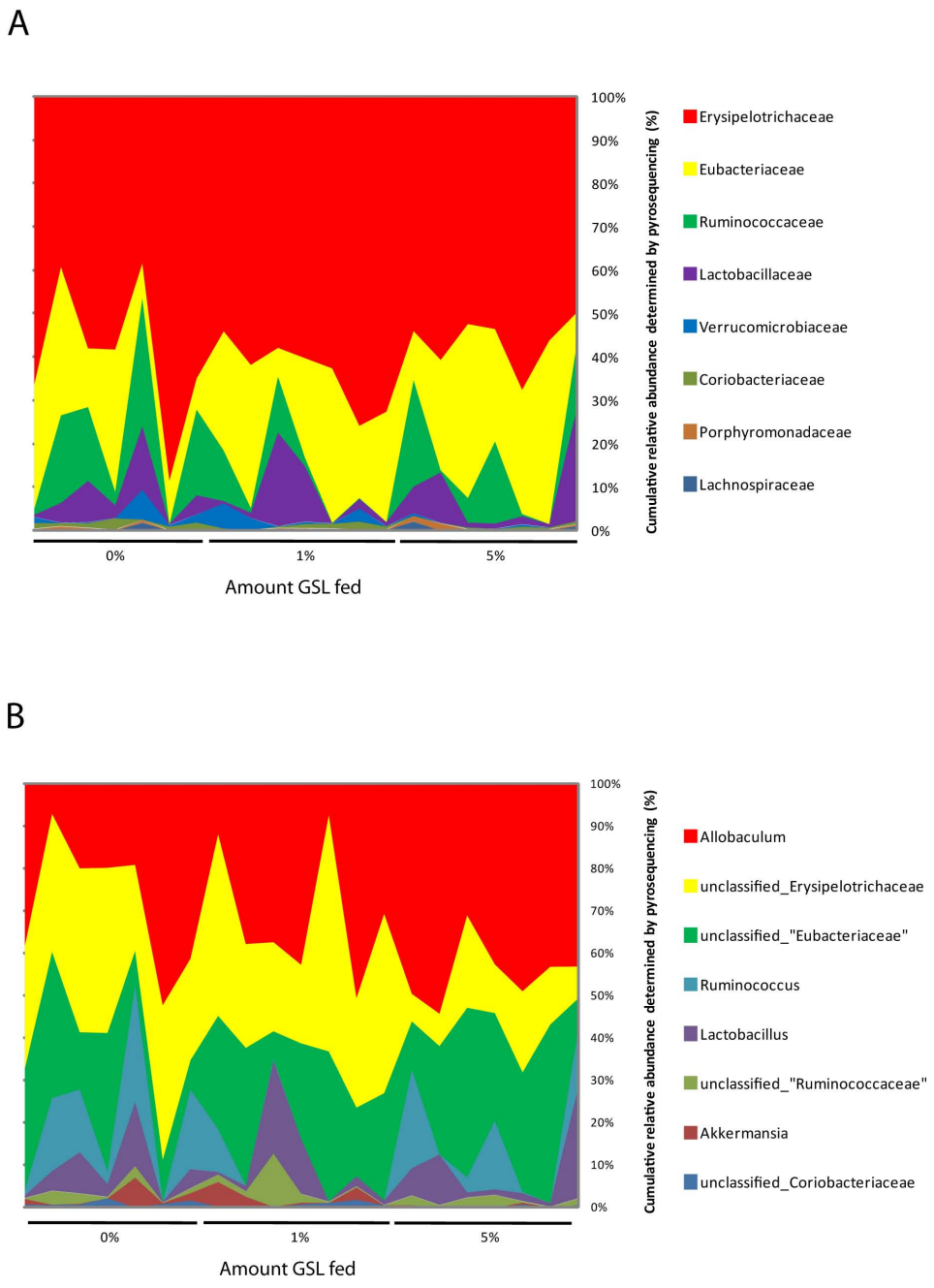

Figure 4.5: **Composition of the fecal microbiota of individual hamsters fed 0%, 1%, and 5% GSL as part of their diet at the family taxonomic level (A) and genus taxonomic level (B).**

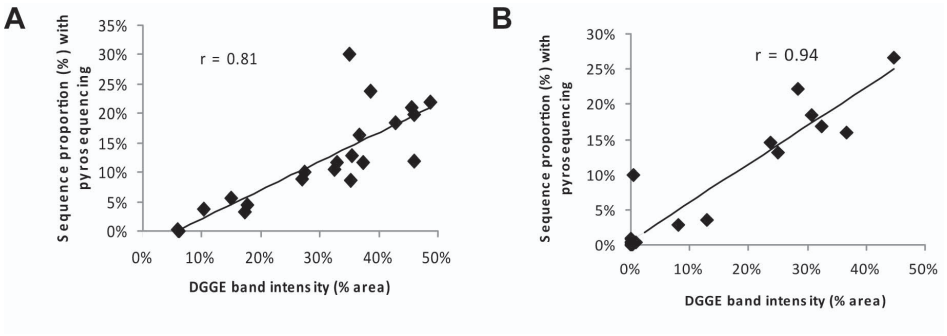

Figure 4.6: **Results obtained by pyrosequencing of 16S rRNA tags and DGGE showed good coherence**. Correlations between relative abundance of bacterial taxa as determined by pyrosequencing and band intensity in DGGE analysis of A) *Allobaculum* and B) *Ruminococcus*.

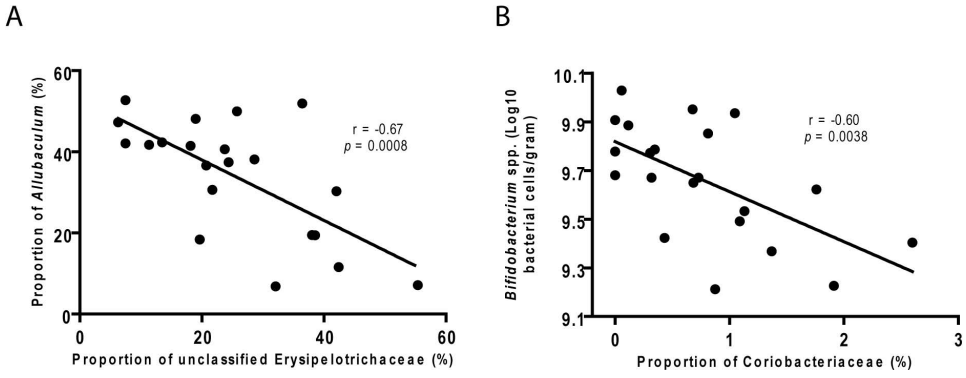

Figure 4.7: **Inverse correlation between the abundance of related bacterial groups in fecal samples of hamsters fed GSL.** A) Abundance of Allobaculum and unclassified Erysipelotrichaceae, which both belong to the family Erysipelotrichaceae and the phylum Firmicutes, as determined by pyrosequencing. B) Abundance of bifidobacteria and Coriobacteria, which both belong to the phylum Actinobacteria, as determined by pyrosequencing (for Coriobacteriaceae) and qRT-PCR (for bifidobacteria). Data from all 21 animals were plotted regardless of the GSL treatments.

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Chapter 5

Increased fecal cholesterol excretion induced by plant sterol esters impacts the gut microbiota composition in Syrian hamsters

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To be submitted.

5.1 Introduction

The mammalian gastrointestinal tract (GIT) is colonized by trillions of microorganism (the gut microbiota), a large fraction of which are bacteria. This microbial community has an extensive impact on host metabolism with important consequences for vertebrate biology (Allen and Torres, 2008, Reddy et al., 1972, Claus et al., 2011). First, the contribution of gut microbes to the utilization and storage of nutrients is a key beneficial trait that underlies host-microbiota symbiosis (Bäckhed et al., 2007, Gordon and Pesti, 1971). Second, the interplay between diet, the gut microbiota, and host metabolism influences the development of metabolic disorders in humans, with various central pathophysiological indicators of disease being reduced in germ-free or antibiotic treated diet-induced and genetic animal models of metabolic diseases (Bäckhed et al., 2004, Ley et al., 2005, Vijay-Kumar et al., 2010, Membrez et al., 2008, Rabot et al., 2010). Therefore, a precise mechanistic understanding of the interrelationships between gut microbes and host metabolism is necessary to gain a more complete understanding of the host-microbial symbiosis in the vertebrate gut and to develop informed strategies to prevent metabolic disorders through modulation of the gut microbiome.

The host lipidome, and specifically cholesterol metabolism, are key factors in the susceptibility to coronary heart disease (CHD), and have been shown to be influenced by the gastrointestinal microbiota (Danielsson and Gustafsson, 1959, Li et al., 2008, Holmes et al., 2011, Swann et al., 2011). With the advent of novel molecular technologies that have enhanced the characterization of microbial communities, associations between host lipid metabolism with bacterial taxa and community structure of the gut microbiome have been identified in both human and animals. Spencer et al. (2011) showed that in humans, levels of Erysipelotrichaceae were directly associated

with changes in liver fat induced through dietary choline depletion, and higher proportions of this bacteria have been identified in morbidly obese individuals (Zhang et al., 2009). This bacterial family was also linked to lipidemic imbalances and other pathological states in mice, and was associated with cholesterol metabolism in a hamster model of hypercholesterolemia (Zhang et al., 2010, Martínez et al., 2009). A second family, the Coriobacteriaceae, has also been repeatedly linked to the host lipidiome. Coriobacteriaceae displayed strong positive correlations with plasma non-HDL in hamsters (Martínez et al., 2009). Claus et al. (2011) confirmed the connection between Coriobacteriaceae, and especially the genus *Eggerthella*, with lipid metabolism in mice, showing positive correlations with hepatic triglyceride levels. The recurrent identification of associations between Coriobacteriaceae and Erysipelotrichaceae, and specific taxa within members of these families, with host lipid and cholesterol phenotypes observed in independent experiments in humans and animals, suggests a genuine link between these bacterial groups and host metabolism (Claus et al., 2011). Despite the strong evidence linking Coriobacteriaceae and Erysipelotrichaceae with host lipid metabolism, causality has not yet been established. Understanding the directionality of the associations between gut microbiota composition and function with host metabolism is crucial to develop therapies that target specific bacterial groups to ameliorate these disorders (Claus et al., 2011). The important similarities between hamsters and humans in terms of their lipid profiles, enzymatic pathways in lipoprotein and bile metabolism, and susceptibility to diet-induced atherosclerosis pose advantages in using these animals to investigate the interplay between cholesterol metabolism and the gut microbiota. However, the impossibility of rearing hamsters germ-free precludes the study of causative relationships between specific bacterial taxa and host cholesterol metabolism using gnotobiotic approaches. Thus, alternative experimental designs need to be developed to provide insight into the interrelationship

between cholesterol metabolism and the gut microbiota.

In this study, we have characterized the interaction between the gut microbiota and cholesterol metabolism in hamsters that received two types of plant sterol esters (PSE) or a control diet (Rasmussen et al., 2006). PSE cause inhibition of cholesterol absorption in the small intestine and consequently lower systemic cholesterol concentrations. The cholesterol-lowering mechanisms of PSE have been fairly well described, and include the displacement of cholesterol in intestinal micelles, co-crystallization between PSE and cholesterol leading to the formation of insoluble crystals, and the impediment of cholesterol hydrolysis by lipases and cholesterol esterases (Heinemann et al., 1991, Ntanios and Jones, 1999, Normén et al., 2000, Trautwein et al., 2003, Brown et al., 2010a,b). Accordingly, the dietary addition of PSE led to a reduction in plasma and liver cholesterol levels, and increased cholesterol by fecal excretion (Rasmussen et al., 2006). In addition to the impact of PSE on the cholesterol metabolism, dietary addition of PSE induced significant shifts in the gut microbiota of the hamsters that showed remarkably tight associations with host cholesterol metabolism. The associations between several bacterial taxa with fecal and biliary cholesterol excretion showed an excellent fit to a sigmoidal inhibitory nonlinear model that describes dose-response relations between bacteria and inhibitory compounds (Mouton et al., 2005, MacDougal, 2006), indicating that host cholesterol excretion can shape microbiome structure through the antimicrobial action of cholesterol.

5.2 Materials and methods

5.2.1 Animal experiments and diets

The fecal samples analyzed here were obtained during a previous study that determined the effect of PSE included in the diet on lipid metabolism of hamsters, and the handling of animals, feed composition, sample collection, and metabolic analysis has been described previously (Rasmussen et al., 2006). Briefly, Bio-F1B male Syrian hamsters (Bio Breeders, MA, USA) were individually caged and randomly assigned to four dietary treatments throughout a 4-week period: a modified AIN-93M diet containing no PSE (C), or containing 5% (w/w) plant sterols esterified with fatty acids from beef tallow (BT), stearic acid (SA), or soybean oil at the expense of dietary fiber. The final energy distribution of each diet was 36% fat, 35% carbohydrate and 29% protein. The animals were housed in a facility with controlled atmosphere (25°C) under 12-hour light:dark cycles, and had access to food and water ad libitum. The animals were euthanized by CO_2 asphyxiation after 4 weeks of dietary intervention. Blood was collected by cardiac puncture, and plasma was obtained by centrifugation (2,000 x g for 30 min at 4°C). Total and HDL cholesterol were enzymatically quantified in the plasma samples. Livers were excised and immediately frozen in liquid nitrogen. Total cholesterol, triglycerides, free cholesterol, esterified cholesterol and phospholipids were measured in the livers. Cholesterol absorption was quantified in fecal samples collected at week 3 with radiolabeled sterols, as previously described (Schneider et al., 2000). The complete fecal output was collected during week 4 and stored frozen (80°C). Fecal quantification of neutral sterols, bile acids, cholesterol, dihydrocholesterol, coprostan-3-one and coprostan-3-ol was performed as previously described (Rasmussen et al., 2006), and fecal samples were subjected to DNA extraction.

5.2.2 Characterization of the fecal microbiota

The gut microbiota was characterized in fecal samples of hamsters fed a control diet (C) (n=7) or plant sterols esterified with stearic acid (SA) (n=9) and beef tallow (BT) (n=6). DNA was extracted by a standard phenol-chloroform extraction method in combination with enzymatic and mechanical cell lysis (Martínez et al., 2009). The fecal microbial community was characterized by massive parallel sequencing of the V2-V3 region of the 16S rRNA gene using the Roche Genome Sequencer GS-FLX (454 Life Sciences) (Martínez et al., 2009). The samples were sequenced with the forward primer (A-338F) 5-gcctccctcgcgccatcagACTCCTACGGGAGGCAGCAG-3 and the reverse primer (B-518R) 5-gccttgccagcccgctcagNNNNNNNNATTACCGCGGCTGC-TGG-3 (with the A and B adaptors indicated in lower case, and an eight nucleotide barcode shown as Ns).

The resulting sequences were quality controlled using the QIIME pipeline (Caporaso et al., 2010). Sequences <150 bp or >350 bp in length were removed, as well as sequences containing one or more ambiguous nucleotides or mismatches to the primer or barcode, sequences with a quality score below 25, and with homopolymer runs longer than 6 bp. Chimera removal was performed using the Blast Fragments algorithm in QIIME. An average of 1,700 sequences per sample were obtained after quality control. Taxonomical characterization of the sequences was done with the Classifier tool from the Ribosomal Database Project (RDP) (Wang et al., 2007), which classified the sequences from the phylum to the genus level. Additionally, an Operational Taxonomic Unit (OTU) based approach with 97% sequence similarity cutoff at a lower taxonomic level. OTUs were generated by aligning the quality-controlled sequences with the Infernal Alignment algorithm of RDP, followed by clustering with the Complete Linkage Clustering tool of RDP. OTUs determined to be significantly affected by plant

sterol consumption or associated with host physiological parameters were confirmed using BLASTn. For this purpose, 5 representative sequences of the selected OTUs were taxonomically assigned and aligned by ClustalW within their respective phylum. Phylogenetic trees were constructed for the sequences belonging to each phylum with the Neighbor-joining algorithm and distance matrices were generated (MEGA 4.0) (Tamura et al., 2007). OTUs were re-assigned with sequences with >97% similarity, and consensus sequences were generated for each new OTU. The consensus sequences were aligned in BioEdit (Hall, 1999) with the BLASTn algorithm against a local database of the entire dataset containing the sequences generated in this study. Sequences that showed >97% similarity and at least 95% overlap were considered to belong to the OTU. UniFrac analysis was performed using the QIIME pipeline to investigate the beta-diversity of the samples.

5.2.3 *In vitro* inhibition bacterial assays

Several bacterial strains originating from the mammalian gastrointestinal tract were used to test the bacteriostatic effects of cholesterol, cholesteryl-linoleate, and the PSE β-sitosteryl-stearate stigmastanyl-stearate when included incorporated into micelles and added to the medium. The bacteria used were: *Bifidobacterium longum* subsp. *infantis* ATCC15697^{T} (in MRS medium supplemented with 0.5 mg/L L-cysteine), *Lactobacillus reuteri* Lpuph-1 (in MRS medium), *Eggerthella lenta* ATCC 25559^{T} (in MRS medium), *Slackia heliotrinireducens* ATCC 29202T, *Collinsella intestinalis* ATCC 13228^{T} (in Peptone Yeast Glucose medium), two human isolates of *Collinsella aerofaciens* KD-D8-5 and IM-D3-18 (in PYG medium), *Clostridium histolyticum* ATCC 19401^{T} (in PYG medium). All media were pre-reduced for 24 h, and cultures were grown at 37°C for 18 h under anaerobic conditions in a Bactron IV

Anaerobic Chamber (Shel Lab).

Micelles containing cholesterol (¿98% purity, Sigma), cholesteryl-linoleate (¿99% purity Sigma), stigmastanol (>95% purity, Sigma) or PSE that were synthesized as previously described (Brown et al., 2010b). Enough chloroform was used to solubilize 161 mg of cholesterol, cholesterol-linoleate, or PSE. Separately, chloroform was added to 221 mg of lecithin until solubilization was reached. 48 μl of the sterol containing solutions were mixed with 41 μl of the lecithin solution, after which the mixture was flushed with nitrogen to remove the chloroform. The resulting mixture of lecithin and sterols was suspended in 10 ml of a 1x10-6 mM solution of bile salt sodium taurocholate (>97% purity, Sigma) in distilled water. Then, sonication was performed for 3-6 min with 30% amplitude using a Branson 450 Sonifier (Danbury, USA). The resulting solutions were filter sterilized with 0.45 μm pore filters (Fisherbrand, Fisher Scientific). Given the bactericidal/bacteriostatic capacity of bile acids, micelles containing bile acids but no sterols were used as control (Percy-Robb and Collee, 1972). 500 μl of pre-reduced micelle suspensions (control) were added to 500 μl of the pre-reduced double-concentrated ($2X$) media. The mixtures were inoculated with 5 μl of a 1:10 dilution of the bacterial cultures. Cultures were incubated in anaerobiosis at 37°C for 12 h, and spectrophotometric optical density (OD) quantifications were performed at 600 nm of wavelength (BioMate3, Thermo Scientific). The experiments were preformed in triplicate.

5.2.4 Statistical analysis

The impact of the treatments on the microbiota was analyzed by one-way ANOVAs and Tukeys post-hoc tests. Correlations between bacterial groups and physiological measurements were assessed with Pearsons correlations and non-linear regressions.

The non-linear model used was the four-parameter sigmoidal inhibitory model commonly used to describe dose-response relations between bacteria and inhibitory compounds. The model is represented by equation (1) (Mouton et al., 2005, MacDougal, 2006), where X represents the metabolic parameter, Y the abundance of the bacterial taxon, Yo and Ymax are the minimum and maximum effects, respectively EC_{50} is the concentration where 50% of the maximum effect is measured, and the slope is the sigmoidicity coefficient.

$$(1) \qquad Y = Y_o + \frac{Y_{max} * X^{slope}}{EC_{50}^{slope} + X^{slope}}$$

The statistical analyses described above were performed using GraphPad Prism version 5.0 (GraphPad Software, USA). $P<0.05$ in the ANOVAs, and correlation coefficients $r>0.60$ (in absolute value) were considered significant. Results were expressed as mean ± SD unless otherwise stated.

5.3 Results

5.3.1 Dietary supplementation with 5% plant sterols induced substantial alterations of the fecal microbiota of hamsters

454 pyrosequencing was used to characterize the fecal microbiota of hamsters fed a control diet, or a diet supplemented with 5% plant sterols esterified with stearic acid (SA) or beef tallow (BT). The inclusion of PSE in the diet, in particular SA, had extensive effects on the fecal microbiota composition. UniFrac analysis revealed that the fecal communities of hamsters fed the SA diet clustered separate from those of

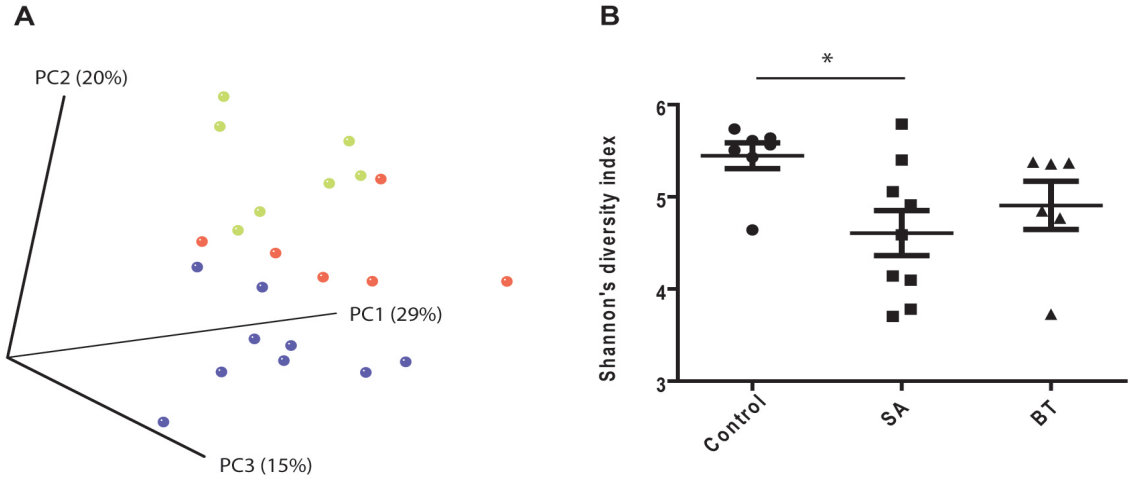

Figure 5.1: **Alpha- and beta- diversity measurements of the fecal bacterial communities.** Principal coordinate analysis based on Unifrac distances, segregated fecal microbial communities of hamsters fed plant sterols esterified with stearic acid (blue) and beef tallow (red) from animals fed a control diet (green) (A). Shannon's diversity index (B). $*$ $P<0.05$.

the control group (Figure 5.1 A). Analysis of Shannon's diversity coefficients revealed that dietary addition of SA reduced the diversity of the fecal microbiota when compared to control ($P<0.05$), and that although not significant, BT had a tendency to also decrease diversity (Figure 5.1 B).

Dietary PSE significantly modulated the gut microbiota at all taxonomic levels (Table 5.1). PSE had a dramatic effect on the abundance of the phylum Actinobacteria, and especially on unclassified members of the family Coriobacteriaceae, which dropped to negligible proportions when SA was fed and were strongly reduced in the BT group. Several OTUs belonging to this family were reduced through PSE (Table 5.1, Supplementary Figure 5.6), with an OTU (OTU1) closely related to the species *Eggerthella lenta* being the most numerical dominant.

SA induced a significant increase in the phylum Firmicutes, and the relative propor-

Table 5.1: **Abundance of fecal bacterial taxa of hamsters fed a control diet, or diets enriched in plant sterol esters.** The taxa presented were significantly affected by plant sterols esterified with stearic acid (SA) or beef tallow (BT), or were determined to be associated with host metabolic markers of the lipid metabolism

	Control	SA	BT	ANOVA P-value
Phylum				
Actinobacteria	16.55 ± 7.27	3.24 ± 1.90[c]	8.37 ± 5.04[a]	0.0002
Firmicutes	72.88 ± 5.50	85.07 ± 7.25[bd]	76.44 ± 4.90	0.0025
Family				
Coriobacteriaceae	11.63 ± 7.30	0.88 ± 0.49[c]	2.71 ± 2.32[b]	0.0002
Eubacteriaceae	0.02 ± 0.05	10.85 ± 11.58[ad]	0.61 ± 1.32	0.0156
Genus				
Unclassified Coriobacteriaceae	11.57 ± 7.30	0.87 ± 0.49[c]	2.66 ± 2.27[b]	0.0002
Unclassified Erysipelotrichaceae	1.08 ± 0.30[a]	0.66 ± 0.24	0.76 ± 0.40	0.0409
Unclassified Eubacteriaceae	0.02 ± 0.05	10.85 ± 11.58[e]	0.61 ± 1.32	<0.0001
OTUs				
OTU1 (*Eggerthella lenta* 97%)	6.98 ± 7.46	0.24 ± 0.31[a]	0.04 ± 0.07[a]	0.0078
OTU2 (*Gordonibacter pamelaeae* 93%)	2.05 ± 0.76	0.12 ± 0.10[c]	0.81 ± 1.19[a]	0.0002
OTU4 (*Slackia heliotrinireducens* 97%)	1.22 ± 0.93	0.03 ± 0.10[b]	0.24 ± 0.30[a]	0.0011
OTU8 (*Allobaculum stercoricanis* 91%)	6.56 ± 4.80	1.48 ± 2.24[a]	2.71 ± 2.51	0.0205
OTU9 (*Allobaculum stercoricanis* 86%)	3.30 ± 3.19	0.15 ± 0.14[b]	0.31 ± 0.28[a]	0.0051
OTU10 (*Eubacterium cylindroides* 87%)	1.03 ± 1.00	0.08 ± 0.07[a]	0.28 ± 0.23	0.0125
OTU12 (*Allobaculum stercoricanis* 94%)	3.14 ± 1.55	0.71 ± 0.86[b]	1.43 ± 1.08[a]	0.0021
OTU13 (*Eubacterium biforme* 87%)	0.52 ± 0.25	0.09 ± 0.09[b]	0.22 ± 0.40	0.0100
OTU15 (*Clostridium sufflavum* 90%)	0.91 ± 0.67	0.20 ± 0.29[a]	0.29 ± 0.21[a]	0.0105
OTU16 (*Eubacterium limosum* 93%)	0.04 ± 0.06	8.55 ± 12.71	0.62 ± 1.49	0.0972

[a] P<0.05 compared to Control.
[b] P<0.01 compared to Control.
[c] P<0.001 compared to Control.
[d] P<0.05 compared to BT.
[e] P<0.01 compared to BT.

tions of Actinobacteria and Firmicutes in the animals showed a strong negative correlation (r=-0.61, P<0.01), suggesting that the increase in Firmicutes induced through PSE was at the expense of Actinobacteria. Members of the Eubacteriaceae family did account for the increase in Firmicutes in the SA group (Table 5.1). However, these shifts could not be traced to lower taxonomic levels, suggesting that several distinct taxa increased at the genus and species level. Although total Firmicutes showed an increase with SA, several OTUs belonging to this phylum did not follow the general trend and instead showed a significant decrease (OTUs 12, 13, and 15) (Table 5.1, Supplementary Figure 5.7). In additions, unclassified members of the Family Erysipelotrichaceae were also reduced by both PSE.

5.3.2 Correlation analysis showed a highly interlinked relationship between the microbiome and the host lipidome

The feeding of PSE induced extensive changes in the lipid and cholesterol metabolism of the hamsters (Rasmussen et al., 2006), and many of the individual metabolites were highly interrelated with each other (Figure 5.2 A). One of the goals of the present study was to investigate whether these effects were associated with specific bacterial groups in the gastrointestinal tract (GIT). Linear correlations and non-linear regression models were used to explore these host-microbiota associations. This analysis revealed remarkable links between the microbiome and host metabolism at all taxonomic levels (Figure 5.2 B). The tightest associations were observed within the phylum Actionbacteria, and especially the family Coriobacteriaceae, which showed remarkable associations with cholesterol absorption (r=0.75, P<0.0001), whole body cholesterol synthesis (r=-0.75, P<0.0001), fecal biliary cholesterol excretion (r=-0.75,

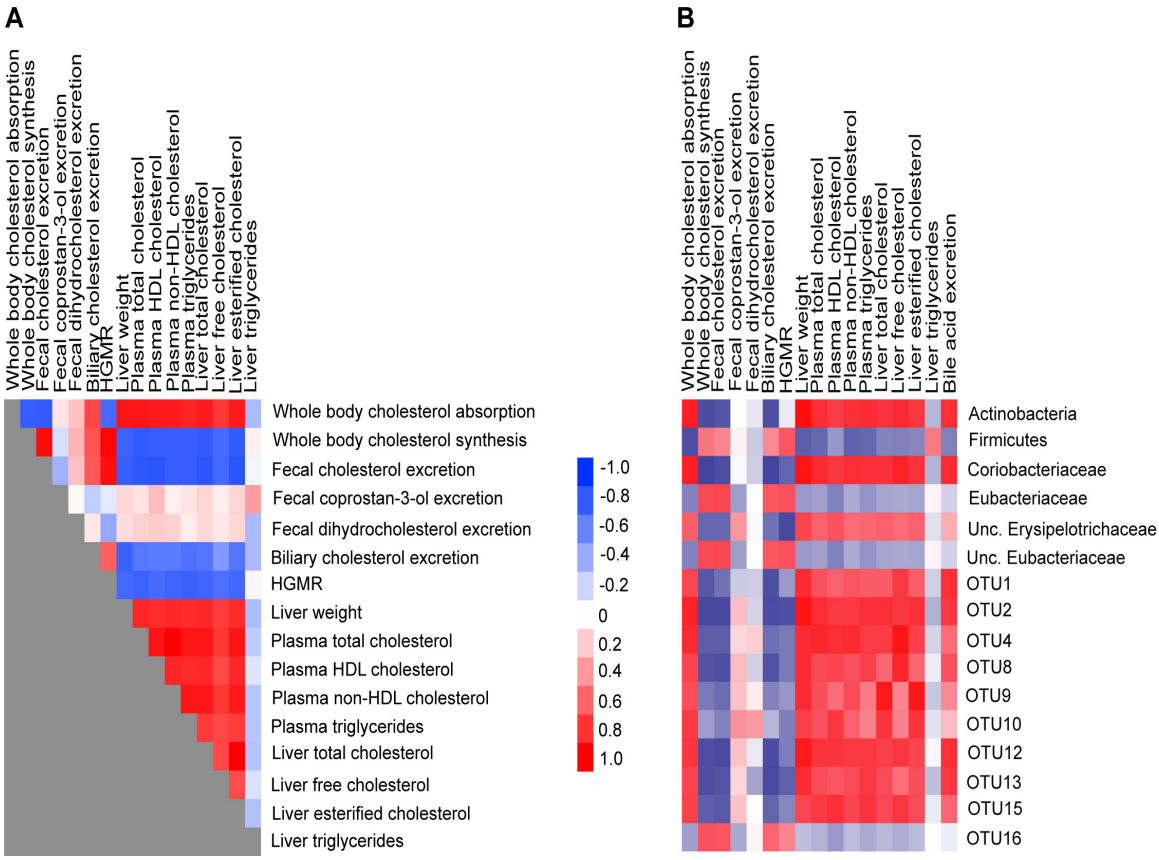

Figure 5.2: **Associations among host markers of the lipid metabolism, and with fecal bacterial populations.** Heatmap displaying correlation coefficients among markers of the host lipid metabolism (A), and between host lipid profile and abundance of fecal bacterial populations (B). Pearson's r correlation and the corresponding.

P<0.0001), liver free cholesterol (r=0.73, P<0.0001), plasma non-HDL cholesterol (r=0.68, P=0.0005), and liver weight (r=0.82, P<0.0001), among others. Therefore, this study confirmed the close associations between Coriobacteriaceae and plasma non-HDL cholesterol and cholesterol absorption that were observed in our previous study (Martínez et al., 2009), and identified additional connections between lipid metabolism and this bacterial taxa. Several OTUs within the Coriobacteriaceae shared the high associations observed for the whole family (Figure 5.2 B).

Total Firmicutes and unclassified Erysipelotrichaceae also displayed significant associ-

ations with host lipidemic markers, although they were less pronounced than the ones determined for the Coriobacteriaceae, with the exception of fecal total sterols, which displayed a high negative associations with unclassified Erysipelotrichaceae (r=-0.88, P<0.0001). Several OTUs classified as Firmicutes related to described species within the genera *Allobaculum*, *Eubacterium*, and *Clostridium* (albeit with low 16S rRNA homologies) showed significant associations with host lipid metabolism. Multivariate analysis also identified *Allobaculum*, Coriobacteriaceae and *Eggerthella* as the fundamental bacterial groups linked to the cholesterol metabolism (P=0.06), and determined cholesterol synthesis to be the main metabolic marker involved in this association.

5.3.3 The data suggested that the improvements in the host lipid metabolism were independent from the gut microbiota

Coprostan-3-one, coprostan-3-ol and dihydrocholesterol are products of bacterial fermentation of cholesterol in the GIT, and are to a large degree excreted and could therefore contribute to the reduction in host cholesterol levels (Schneider et al., 2000). However, fecal concentrations of these cholesterol derivatives were not affected by feeding PSE nor did they correlate with any of the host lipid metabolism markers tested (Figure 5.2 A). In addition, the correlation analysis did not provide evidence for an association between the coprostan-3-one, coprostan-3-ol, and dihydrocholesterol and specific bacterial members of the gut microbiota (Figure 5.2). These findings indicate that PSE induced shifts of the gut microbiome did not impact bacterial cholesterol metabolites in the gastrointestinal lumen, nor was the bacterial action on the cholesterol pool responsible for the improvements observed in the host lipid

metabolism.

5.3.4 Associations between bacterial taxa and cholesterol synthesis, fecal biliary cholesterol excretion, and fecal total cholesterol excretion fit a mathematical model of bacterial inhibition

Upon visual inspection of the correlation graphs between bacterial taxa with host cholesterol synthesis and excretion, these negative interactions were clearly not linear but rather had an exponential relationship. More importantly, these associations resembled the dose-response curves in microbial antibiotic assays. We therefore tested how bacterial proportions and cholesterol synthesis/excretion fitted a four-parameter sigmoid model for bacterial inhibition used to describe dose-response relations between bacteria and inhibitory compounds (Mouton et al., 2005, MacDougal, 2006). Graphs presented in Figure5.3 display the host metabolite-microbial relationships with the tightest associations, and reveal that the empirical data obtained in the hamster experiments is an excellent fit to the inhibitory model. For example, the associations between Coriobacteriaceae and host cholesterol synthesis, fecal biliary cholesterol excretion, and fecal cholesterol excretion fitted the model with regression coefficients of R of -0.93, -0.87, and -0.91 respectively. The model was also an excellent fit for the data obtained for OTU1, which is related to Eggerthella lenta. Interestingly, the fecal proportions of OTU13 that was classified as a member of the Firmicutes phylum also showed an association with an excellent fit to the model. The fact that these bacterial taxa displayed associations to fecal cholesterol concentrations in the feces that fit an inhibition model suggests a bactericidal/bacteriostatic role of cholesterol excreted through the bile in hamsters.

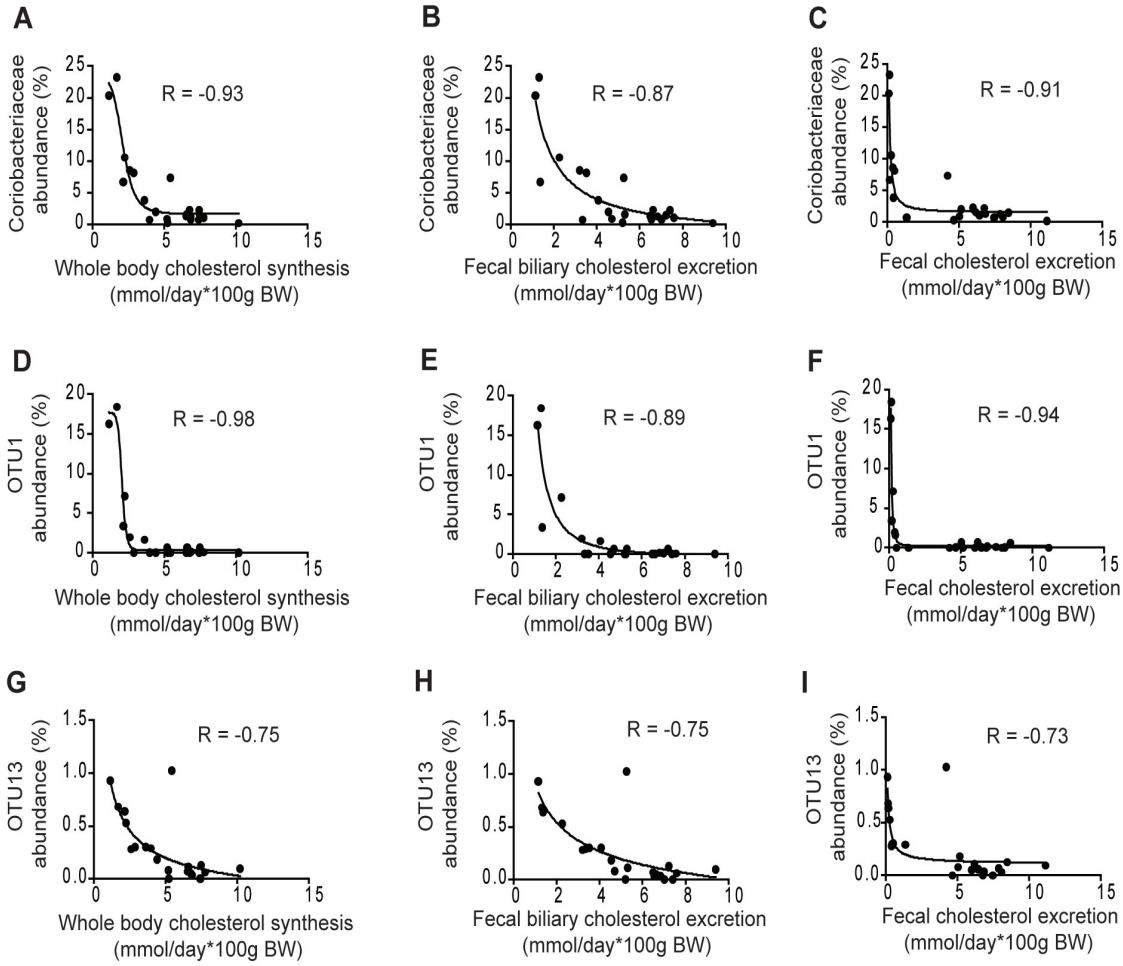

Figure 5.3: **Inhibitory associations between fecal bacterial populations and markers of host cholesterol metabolism.** Four-parameter sigmoidal inhibitory regressions between fecal abundance of Coriobacteriaceae with whole body cholesterol synthesis (A), fecal biliary cholesterol excretion (B), fecal cholesterol excretion (C), between the proportions of OTU1 with whole body cholesterol synthesis (D), fecal biliary cholesterol excretion (E), fecal cholesterol excretion (F), and between the abundance of OTU13 with whole body cholesterol synthesis (G), fecal biliary cholesterol excretion (H), fecal cholesterol excretion (I).

5.3.5 Cholesteryl-linoleate but not PSE inhibit growth of gut bacteria in vitro

To provide further insight into the associations between bacteria and cholesterol metabolism, we determined the ability of cholesterol, cholesteryl-linoleate, stigmastanol and β-sitosterol to inhibit bacterial growth when included in micelles formed with bile acids and lecithin which are natural components of micelles in the gut (Mukhopadhyay and Maitra, 2004). We tested for the inhibition of seven strains of gut bacteria belonging to the Actionbacteria and Firmicutes, as members of these phyla were most affected in our animal experiments. Three bacteria out of the seven strains

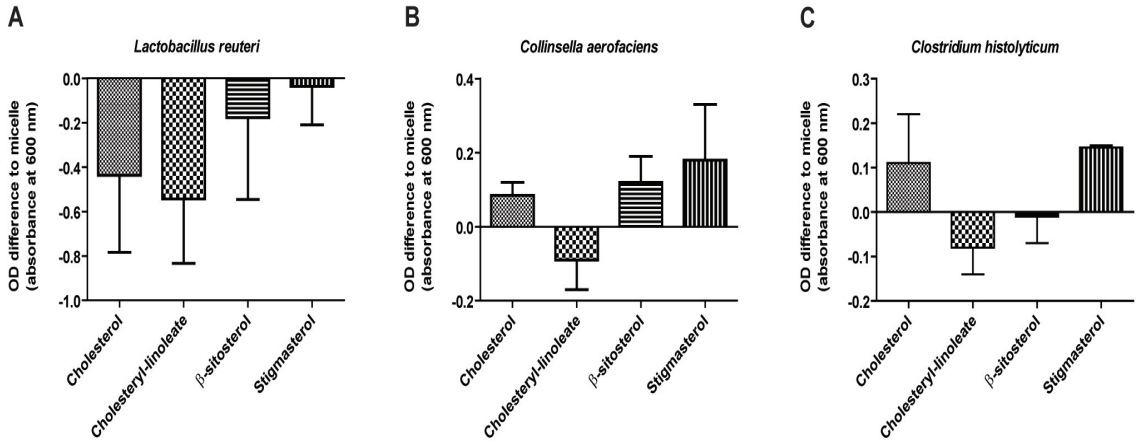

Figure 5.4: ***In vitro* inhibition of fecal bacterial isolates by sterols.** Optical density difference between micelles containing cholesterol, cholesteryl-linoleate, β-sitosterol, and stigmasterol compared to control micelles without sterols for the gut bacterial isolates *Lactobacillus reuteri* (A), *Collinsella aerofaciens* (B), and *Clostridium histolyticum* (C).

tested were inhibited by micelles that contained cholesteryl-linoleate when compared to control micelles with only lecithin and bile acid, *Lactobacillus reuteri*, *Clostridium histolyticum*, and *Collinsella aerofaciens* (Figure 5.4). Growth of *Lactobacillus reuteri* was also inhibited by micelles containing cholesterol. Most importantly, inclusion of

PSE to the micelles had either no or only a minor inhibitory action.

5.4 Discussion

In this study, we characterized the interplay between the gut microbiota and cholesterol metabolism in hamsters that received plasma cholesterol-lowering PSE in order to gain mechanistic information on cause and effect. The data revealed that the modifications of host cholesterol metabolism induced through PSE were tightly associated with specific alterations of the gut microbiota over the entire taxonomic scale. The strongest associations were identified between bacteria of the Coriobacteriaceae and Erysipelotrichaceae and the host lipidome. Therefore, our findings are in accordance with previous research that revealed connections between these bacterial families and host metabolism in mice, hamsters, and humans (Claus et al., 2011, Zhang et al., 2009, 2010). The study also confirmed our previous findings in hamsters fed grain sorghum lipids (GSL), which also displayed strong positive and negative associations between Coriobacteriaceae and plasma HDL-cholesterol levels and cholesterol absorption, respectively (Martínez et al., 2009). The strong associations between bacterial taxa in the gut and the host lipidome are of significant interest to the field as they suggest that bacteria impact host metabolism and could constitute potential pharmaceutical targets. However, this study provided evidence indicating that the shifts in gut microbiota composition associated with host cholesterol metabolism are not causal but a response to the latter.

Based on the data, modulations of the cholesterol metabolism by PSE are likely independent of the gut microbiome. The ability of PSE to preclude cholesterol is based on physico-chemical interactions and bacteria have not been described as a contributing factor for reduced cholesterol absorption (Trautwein et al., 2003). In addition, PSE

has been reported to have an antimicrobial effect nor did they show inhibitory capacity in our in vitro assays. Therefore, it is unlikely that a direct antimicrobial effect of PSE accounted for the significant alterations of the gut microbiota, especially the drastic decrease of Coriabacteriaceae. The data also revealed that the fecal levels of coprostan-3-one, coprostan-3-ol and dihydrocholesterol were not affected by dietary PSE, and were not correlated to the metabolic improvements of the hamsters. In contrast, fecal cholesterol showed highly significant associations with the improvements in the host lipid metabolism. Conversely, the data provided evidence that suggest that changes in host cholesterol metabolism induced through dietary PSE were the main drivers of the observed alteration of the gut microbiome composition. The mechanisms in which it is envisioned that these associations happened are summarized in Figure 5.5. We propose that the intake of PSE decreased plasma and liver cholesterol levels though an inhibition of both dietary and biliary cholesterol absorption, which consecutively increased fecal cholesterol excretion (Figure 5.5 A). In order to maintain cholesterol homeostasis, the host compensated for the lower cholesterol absorption by increasing cholesterol synthesis, which resulted in increased levels of cholesterol excreted through the bile and feces. Enhanced biliary cholesterol excretion into the intestinal lumen, together with decreased cholesterol absorption, resulted in higher concentrations of free and esterified cholesterol in the gastrointestinal tract. We propose that the antimicrobial action of these cholesterol derivatives inhibited specific bacterial taxa in the gut, and thereby led to strong associations between the gut microbiome and alterations of host metabolism (Figure 5.5 B).

There are several lines of evidence that support the model presented in Figure 5. First, cholesterol and its derivatives have been shown to inhibit bacterial growth at the epithelial linings of the nose and eye (Do et al., 2008, Marquart et al., 2007). The in vitro assays performed in this study confirmed the antibacterial effect of

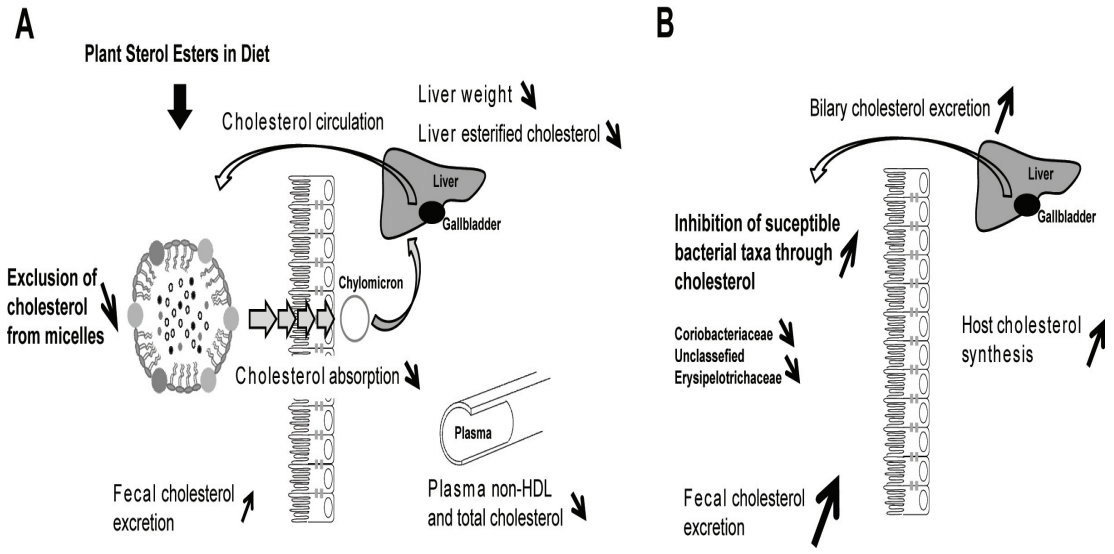

Figure 5.5: **Model depicting the impact of plant sterol esters on the host lipid metabolism and on the gut microbiota.** The inclusion of dietary plant sterol esters (PSE) precludes the incorporation of cholesterol into the micelles in the small intestine, lowering cholesterol absorption and increasing cholesterol excretion, causing reductions of plasma non-HDL, liver weight and hepatic liver esterified cholesterol (A). The decreased cholesterol absorption is compensated by increased hepatic cholesterol synthesis and biliary cholesterol excretion by the host. The higher concentrations of fecal cholesterol coupled with the inhibitory capacity of cholesterol result in the decreased abundance of bacterial populations in the gastrointestinal tract, including Coriobacteriaceae and unclassified Erysipelotrichaceae (B).

cholesteryl-linoleate on gut bacterial isolates. Although the observed inhibitory effect by cholesteryl-linoleate in our experiments was not pronounced, in a highly competitive environment such as the GIT, this seemingly small inhibition could potentially translate into a significant loss of fitness for the organism in the GIT.

Table 5.2: **Correlations between proportions of bacterial taxa and markers of lipid metabolism.**

	Cholesterol absorption (μ/day* 100g BW)	Whole body cholesterol synthesis (μ/day* 100g BW)	Fecal cholesterol excretion (μ/day*100g BW)	Fecal coprostan-3-ol excretion (μ/day*100g BW)	Fecal dihydrocholesterol excretion (μ/day* 100g BW)	Plasma total cholesterol (mg/dl)	Plasma HDL (mg/dl)	Plasma non-HDL (mg/dl)	Bile acid excretion (μ/day* 100g BW)
Phylum									
Actinobacteria	r = 0.75	r = -0.69	r = -0.65	r = 0.01	r = -0.11	r = 0.68	r = 0.62	r = 0.67	r = 0.68
	P<0.0001	P = 0.0003	P = 0.0010	P = 0.9347	P = 0.6226	P = 0.0005	P = 0.0022	P = 0.0006	P = 0.0005
Firmicutes	r = -0.68	r = 0.43	r = 0.38	r = 0.05	r = -0.23	r = -0.59	r = -0.43	r = -0.61	r = -0.50
	P = 0.0004	P = 0.0476	P = 0.0827	P = 0.8252	P = 0.3008	P = 0.0042	P = 0.0432	P = 0.0025	P = 0.0191
Family									
Coriobacteriaceae	r = 0.75	r = -0.75	r = -0.68	r = -0.02	r = -0.22	r = 0.69	r = 0.64	r = 0.68	r = 0.70
	P<0.0001	P<0.0001	P= 0.0005	P = 0.7268	P = 0.4956	P = 0.0004	P = 0.0012	P = 0.0005	P = 0.0003
Eubacteriaceae	r = -0.49	r = 0.57	r = 0.58	r = -0.41	r = -0.01	r = -0.40	r = -0.50	r = -0.35	r = -0.20
	P = 0.0220	P = 0.0051	P = 0.0048	P = 0.0557	P = 0.9583	P = 0.0674	P = 0.0186	P = 0.1072	P = 0.3630
Genus									
Unclassified	r = 0.50	r = -0.59	r = -0.59	r = 0.33	r = -0.03	r = 0.49	r = 0.54	r = 0.46	r = 0.26
Erysipelotrichaceae	P = 0.0169	P = 0.0038	P = 0.0040	P = 0.1284	P = 0.8969	P = 0.0196	P = 0.0089	P = 0.0308	P = 0.2348
Unclassified	r = -0.49	r = 0.58	r = 0.58	r = -0.41	r = -0.01	r = -0.40	r = -0.50	r = -0.35	r = -0.20
Eubacteriaceae	P = 0.0221	P = 0.0051	P = 0.0048	P = 0.0558	P = 0.9590	P = 0.0675	P = 0.0187	P = 0.1074	P = 0.3630
OTUs									
OTU1	r =0.58	r = -0.64	r = -0.55	r = -0.23	r = -0.22	r = 0.54	r = 0.49	r = 0.53	r = 0.67

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Table 5.2 – continued from previous page

	Cholesterol absorption (μmol/day* 100g BW)	Whole body cholesterol synthesis (μmol/day* 100g BW)	Fecal cholesterol excretion (μmol/day*100g BW)	Fecal coprostan-3-ol excretion (μmol/day*100g BW)	Fecal dihydrocholesterol excretion (μmol/day* 100g BW)	Plasma total cholesterol (mg/dl)	Plasma HDL (mg/dl)	Plasma non-HDL (mg/dl)	Bile acid excretion (μmol/day* 100g BW)
(*Eggerthella lenta* 97%)	P = 0.0049	P = 0.0013	P = 0.0082	P = 0.3062	P = 0.3218	P = 0.1010	P = 0.0195	P = 0.0111	P = 0.0006
OTU2 (*Gordonibacter pamelaeae* 93%)	r = 0.73 P = 0.0001	r = -0.71 P = 0.0002	r = -0.72 P = 0.0002	r = 0.21 P = 0.3565	r = -0.19 P = 0.3885	r = 0.68 P = 0.0005	r = 0.63 P = 0.0016	r = 0.63 P = 0.0006	r = 0.64 P = 0.0013
OTU4 (*Slackia heliotrinireducens* 97%)	r = 0.70 P = 0.0003	r = -0.62 P = 0.0023	r = -0.62 P = 0.0019	r = 0.13 P = 0.5777	r = 0.16 P = 0.4798	r = 0.71 P = 0.0002	r = 0.68 P = 0.0005	r = 0.70 P = 0.0003	r = 0.46 P = 0.0298
OTU8 (*Allobaculum stercoricanis* 91%)	r = 0.59 P = 0.0040	r = -0.66 P = 0.0008	r = -0.67 P = 0.0007	r = 0.22 P = 0.3216	r = -0.20 P = 0.3829	r = 0.57 P = 0.0058	r = 0.59 P = 0.0040	r = 0.54 P = 0.0090	r = 0.54 P = 0.0088
OTU9 (*Allobaculum stercoricanis* 86%)	r = 0.55 P = 0.0074	r = -0.52 P = 0.0124	r = -0.57 P = 0.0058	r = 0.20 P = 0.3716	r = 0.07 P = 0.7642	r = 0.55 P = 0.0078	r = 0.46 P = 0.0298	r = 0.56 P = 0.0068	r = 0.37 P = 0.0868
OTU10 (*Eubacterium cylindroides* 87%)	r = 0.63 P = 0.0017	r = -0.41 P = 0.0600	r = -0.50 P = 0.0168	r = 0.35 P = 0.1084	r = 0.32 P = 0.1480	r = 0.54 P = 0.0094	r = 0.64 P = 0.0014	r = 0.49 P = 0.0199	r = 0.22 P = 0.3193
OTU12	r = 0.64	r = -0.68	r = -0.70	r = 0.20	r = -0.10	r = 0.65	r = 0.65	r = 0.63	r = 0.67

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Table 5.2 – continued from previous page

	Cholesterol absorption (μmol/day* 100g BW)	Whole body cholesterol synthesis (μmol/day* 100g BW)	Fecal cholesterol excretion (μmol/day*100g BW)	Fecal coprostan-3-ol excretion (μmol/day*100g BW)	Fecal dihydrocholesterol excretion (μmol/day* 100g BW)	Plasma total cholesterol (mg/dl)	Plasma HDL (mg/dl)	Plasma non-HDL (mg/dl)	Bile acid excretion (μmol/day* 100g BW)
(*Allobaculum stercoricanis* 94%)	P = 0.0015	P = 0.0005	P = 0.0003	P = 0.3694	P = 0.6597	P = 0.0009	P = 0.0010	P = 0.0016	P = 0.0007
OTU13 (*Eubacterium biforme* 87%)	r = 0.59 P = 0.0040	r = -0.68 P = 0.0005	r = -0.65 P = 0.0010	r = 0.13 P = 0.5682	r = -0.38 P = 0.0773	r = 0.56 P = 0.0062	r = 0.53 P = 0.0118	r = 0.56 P = 0.0072	r = 0.64 P = 0.0015
OTU15 (*Clostridium sufflavum* 90%)	r = 0.60 P = 0.0029	r = -0.62 P = 0.0020	r = -0.63 P = 0.0018	r = 0.20 P = 0.3700	r = -0.00 P = 0.9941	r = 0.63 P = 0.0018	r = 0.67 P = 0.0007	r = 0.59 P = 0.0037	r = 0.48 P = 0.0225
OTU16 (*Eubacterium limosum* 93%)	r = -0.40 P = 0.0684	r = 0.54 P = 0.0090	r = 0.53 P = 0.0117	r = -0.36 P = 0.0959	r = 0.04 P = 0.8672	r = -0.32 P = 0.1487	r = -0.42 P = 0.0546	r = -0.28 P = 0.0210	r = -0.08 P = 0.7318

Interestingly, although an antibacterial capacity of bile acids has been described (Stacey and Webb, 1947) and their excretion is affected by dietary PSE (Rasmussen et al., 2006), we determined only weak correlations between fecal bile acid concentration and bacterial groups (Supplementary Figure 5.3, despite rather high Pearson's r values presented in Table 5.2 and Supplementary Table 5.3). In contrast, we detected that the associations between several bacterial populations (Coriobacteriaceae, three OTUs) with fecal biliary and cholesterol excretion showed a remarkable fit to a mathematical model that describes dose-response relations between bacteria and bactericidal compounds.

Although the results obtained might constitute an example of how host physiology can impact the gut microbiota composition, other host-microbiome interactions have been described in which a causative role of gut bacteria has been identified (Cani et al., 2008, Bäckhed et al., 2007, Ley et al., 2005, Vijay-Kumar et al., 2010). In addition, specific bacterial taxa have been determined to improve lipid markers in the host. Bifidobacteria have been identified to alleviate dyslipidemia and high-fat-induced insulin resistance (Chen et al., 2011, Kondo et al., 2011, Yin et al., 2010). In addition, combinations of probiotic lactobacilli and bifidobacteria strains have been determined to increase plasma HDL in women (Kiessling et al., 2002, Sadrzadeh-Yeganeh et al., 2010). Bifidobacteria had been determined to have a positive association to plasma HDL in our previous hamster study (Martínez et al., 2009). However, this study clearly suggests that interactions between the gut microbiota and host metabolism are bi-directional.

5.5 Conclusions

In this study we determined that PSE extensively impacted the gut microbiota composition without exhibiting antimicrobial activity. In addition, the almost perfect fit of the associations between bacterial populations with fecal cholesterol concentrations to a mathematical model of bacterial inhibition suggest that host metabolism can shape the gut microbiota composition. This study constitutes an important advancement of our understanding of the gut microbiome and its relation to the host, as it identified cholesterol as an important host factor that impacts gut microbiota composition and that might contribute to the maintenance of homeostasis in the gut. The data further supported the notion that dysbiotic alterations of the gut microbiome associated with metabolic disease (Vijay-Kumar et al., 2010, Henao-Mejia et al., 2012) or dietary patterns (Wu et al., 2011, Martínez et al., 2009) could be a response of the metabolic phenotypes of the host. Based on the data obtained in this study, we have to revisit our own hypothesis that Coriobacteriaceae might constitute a therapeutic target for the modulation of host cholesterol metabolism, as it appears that this bacterial group is affected by cholesterol metabolism rather than vice-versa. Research on the directionality of metabolic host-microbiota interactions, such as presented here, can provide important mechanistic understanding necessary to make informed strategies to prevent metabolic disorders by modulating the gut microbiota.

5.6 Supplementary materials

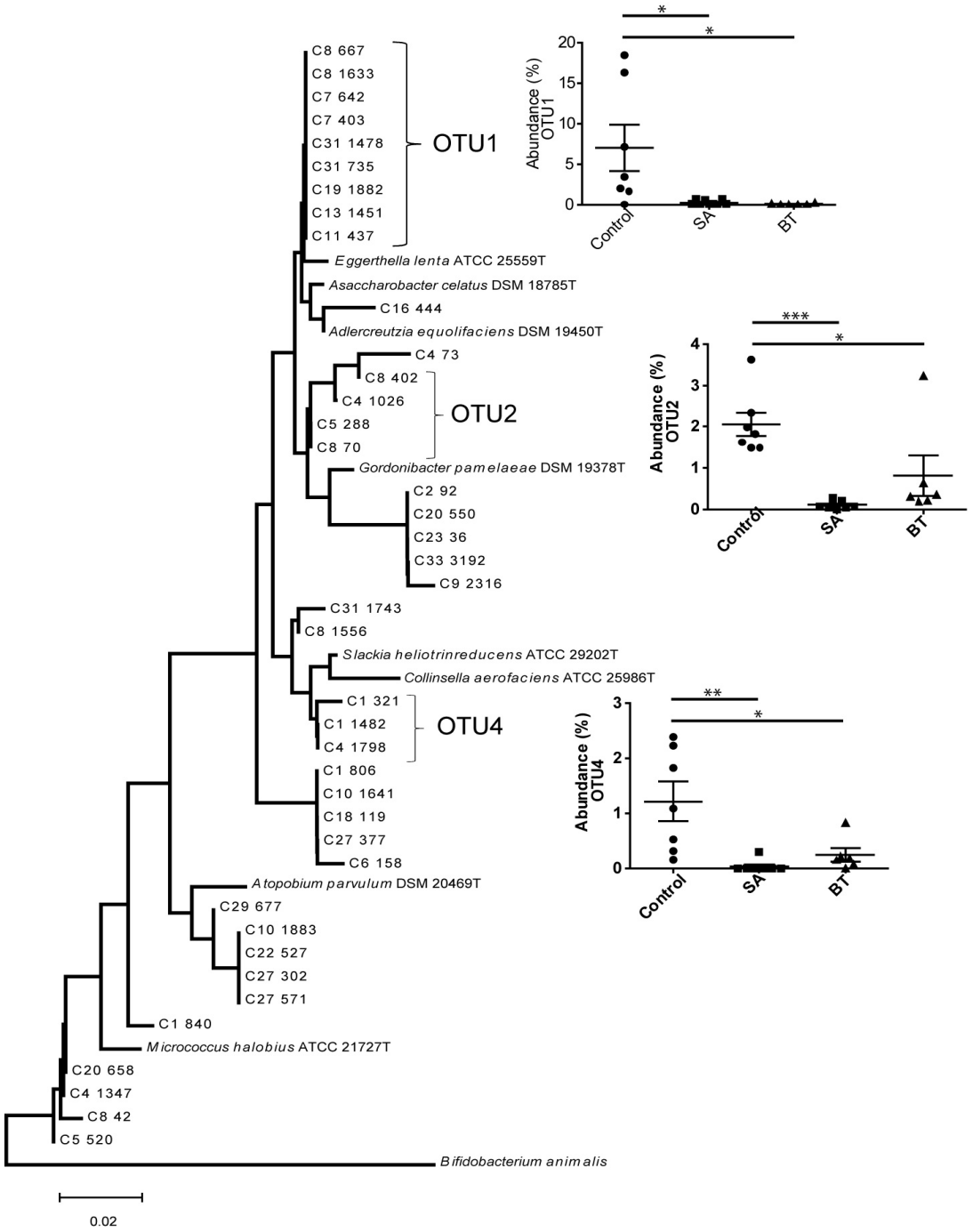

Figure 5.6: **Phylogenetic tree of constructed with the 16S rRNA gene tags generated by pyrosequencing of OTUs belonging to the Coriobacteriaceae family that were significantly affected by dietary plant sterol esters (PSE).** Representative sequences of each OTU were aligned with related type strain 16S rRNA sequences to generate the phylogenetic tree. Consensus sequences were obtained from sequences with >97% similarity, and used as query sequences for local blasts with a database for the tags obtained in the study. The graphs show the proportions of OTUs significantly affected by dietary PSE. $*$ $P<0.05$, $**$ $P<0.01$, $***$ $P<0.001$.

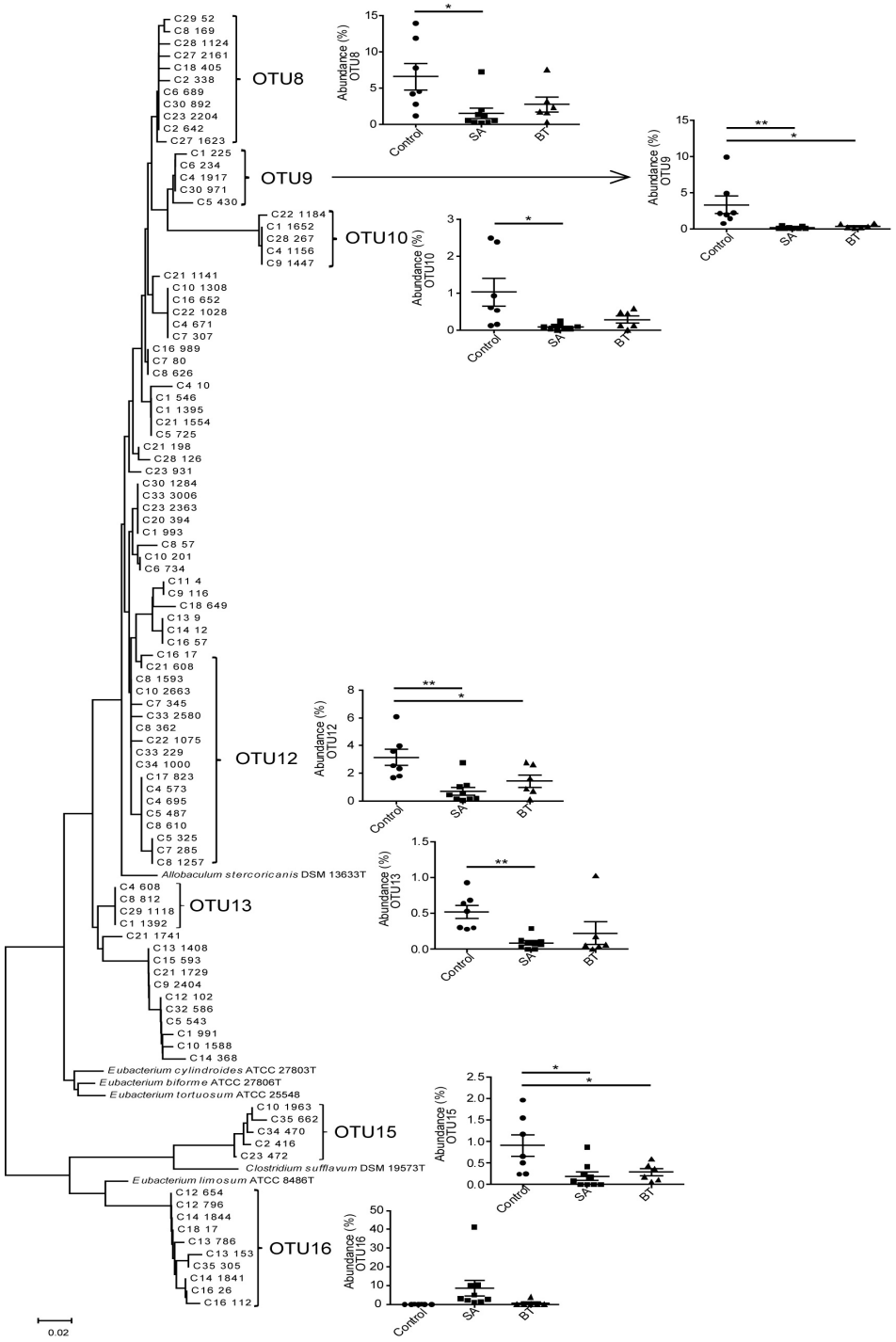

Figure 5.7: **Phylogenetic tree of constructed with the 16S rRNA gene tags generated by pyrosequencing of OTUs belonging to the Eubacteriaceae and Erysipelotrichaceae families that were significantly affected by dietary plant sterol esters (PSE).** Representative sequences of each OTU were aligned with related type strain 16S rRNA sequences to generate the phylogenetic tree. Consensus sequences were obtained from sequences with >97% similarity, and used as query sequences for local blasts with a database for the tags obtained in the study. The graphs show the proportions of OTUs significantly affected by dietary PSE. $*$ $P<0.05$, $**$ $P<0.01$.

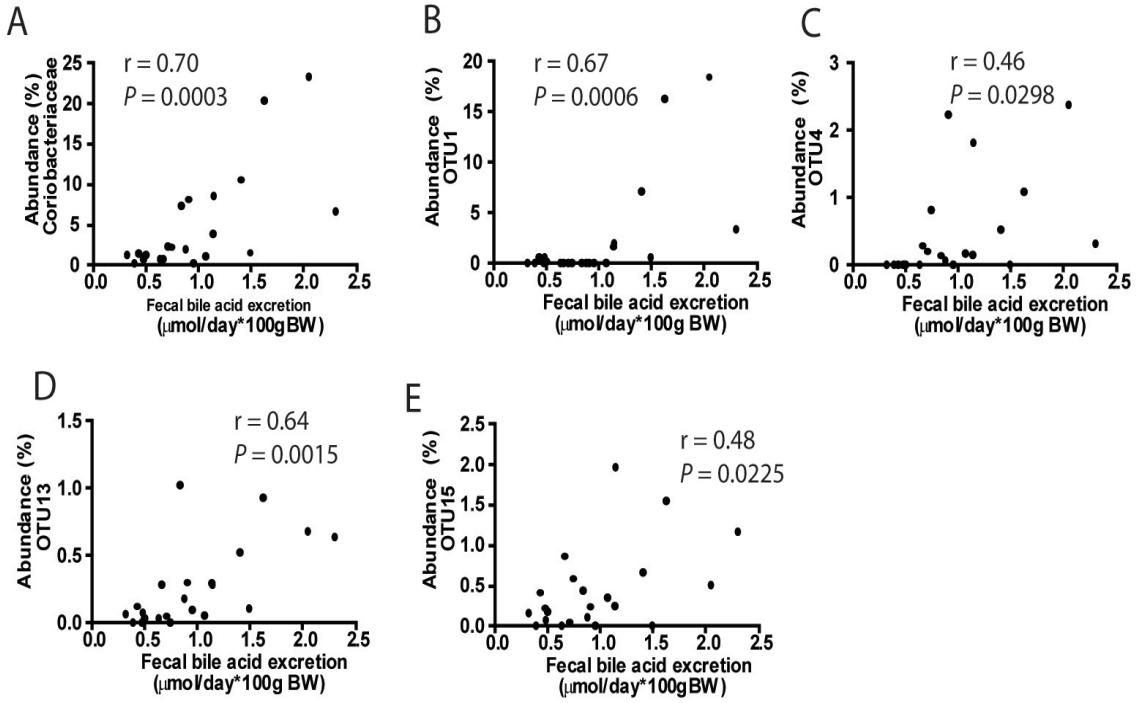

Figure 5.8: **Associations between bile acid excretion and proportions of gut bacterial populations.** Correlations between bile acid excretion and the abundance of Coriobacteriaceae (A), OTU1 (B), OTU4 (C), OTU13 (D), and OTU15 (E).

Table 5.3: **Correlations between proportions of bacterial taxa and markers of lipid metabolism.**

	Plasma triglycerides (mg/dl)	Fecal biliary cholesterol excretion (μ/day* 100g BW)	Liver weight (g)	Liver total cholesterol (mg/g)	Liver free cholesterol (mg/g)	Liver esterified cholesterol (mg/g)	Liver triglycerides (mg/g)
Phylum							
Actinobacteria	r = 0.69	r = -0.68	r = 0.87	r = 0.65	r = 0.70	r = 0.64	r = -0.31
	P = 0.0004	P = 0.0005	P <0.0001	P = 0.0010	P = 0.0003	P = 0.0013	P = 0.1694
Firmicutes	r = -0.59	r = 0.37	r = -0.62	r = -0.49	r = -0.50	r = -0.48	r = 0.42
	P = 0.0039	P = 0.0884	P = 0.0023	P = 0.0208	P = 0.0173	P = 0.0225	P = 0.0498
Family							
Coriobacteriaceae	r = 0.66	r = -0.75	r = 0.82	r = 0.66	r = 0.73	r = 0.65	r = -0.32
	P = 0.0009	P <0.000	P <0.0001	P = 0.0009	P = 0.0001	P = 0.0012	P = 0.1440
Eubacteriaceae	r = -0.44	r = 0.52	r = -0.42	r = -0.37	r = -0.36	r = -0.37	r = 0.04
	P = 0.0396	P = 0.0137	P = 0.522	P = 0.0867	P = 0.0983	P = 0.0928	P = 0.8463
Genus							
Unclassified	r = 0.47	r = -0.55	r = 0.59	r = 0.49	r = 0.46	r = 0.49	r = -0.14
Erysipelotrichaceae	P = 0.0283	P = 0.0081	P = 0.0036	P = 0.0193	P = 0.0322	P = 0.0205	P = 0.5486
Unclassified	r = -0.44	r = 0.52	r = -0.42	r = -0.37	r = -0.36	r = -0.37	r = 0.04
Eubacteriaceae	P = 0.0397	P = 0.0137	P = 0.0524	P = 0.0869	P = 0.0985	P = 0.0930	P = 0.8479
OTUs							
OTU1	r = 0.51	r = -0.67	r = 0.69	r = 0.51	r = 0.63	r = 0.50	r = -0.21
(*Eggerthella lenta* 97%)	P = 0.0145	P = 0.0006	P = 0.0004	P = 0.0145	P = 0.0016	P = 0.0173	P = 0.3562

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Table 5.3 – continued from previous page

	Plasma triglycerides (mg/dl)	Fecal biliary cholesterol excretion (μ/day* 100g BW)	Liver weight (g)	Liver total cholesterol (mg/g)	Liver free cholesterol (mg/g)	Liver esterified cholesterol (mg/g)	Liver triglycerides (mg/g)
OTU2 (*Gordonibacter pamelaeae* 93%)	r = 0.66 P = 0.0009	r = -0.69 P = 0.0004	r = 0.81 P <0.0001	r = 0.67 P = 0.0007	r = 0.64 P = 0.0014	r = 0.66 P = 0.0008	r = -0.33 P = 0.1322
OTU4 (*Slackia heliotrinireducens* 97%)	r = 0.62 P = 0.0022	r = -0.56 P = 0.0064	r = 0.69 P = 0.0004	r = 0.62 P = 0.0022	r = 0.81 P <0.0001	r = 0.60 P = 0.0030	r = -0.19 P = 0.3975
OTU8 (*Allobaculum stercoricanis* 91%)	r = 0.60 P = 0.0031	r = -0.66 P = 0.0008	r = 0.69 P = 0.0003	r = 0.48 P = 0.0261	r = 0.72 P = 0.0002	r = 0.47 P = 0.0285	r = -0.08 P = 0.7224
OTU9 (*Allobaculum stercoricanis* 86%)	r = 0.41 P = 0.0554	r = -0.49 P = 0.0211	r = 0.69 P = 0.0032	r = 0.78 P <0.0001	r = 0.38 P = 0.0830	r = 0.79 P <0.0001	r = -0.24 P = 0.2857
OTU10 (*Eubacterium cylindroides* 87%)	r = 0.39 P = 0.0738	r = -0.31 P = 0.1564	r = 0.60 P = 0.0116	r = 0.65 P = 0.0011	r = 0.39 P = 0.0718	r = 0.65 P = 0.0010	r = -0.13 P = 0.5566
OTU12 (*Allobaculum stercoricanis* 94%)	r = 0.63 P = 0.0015	r = -0.69 P = 0.0004	r = 0.78 P <0.0001	r = 0.60 P = 0.0030	r = 0.64 P = 0.0013	r = 0.59 P = 0.0037	r = -0.04 P = 0.8442
OTU13 (*Eubacterium biforme* 87%)	r = 0.63 P = 0.0019	r = -0.70 P = 0.0003	r = 0.69 P = 0.0004	r = 0.56 P = 0.0073	r = 0.45 P = 0.0365	r = 0.55 P = 0.0076	r = -0.27 P = 0.2174

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Table 5.3 – continued from previous page

	Plasma triglycerides (mg/dl)	Fecal biliary cholesterol excretion (μ/day* 100g BW)	Liver weight (g)	Liver total cholesterol (mg/g)	Liver free cholesterol (mg/g)	Liver esterified cholesterol (mg/g)	Liver triglycerides (mg/g)
OTU15 (*Clostridium sufflavum* 90%)	r = 0.65 P = 0.0010	r = -0.60 P = 0.0030	r = 0.58 P = 0.0049	r = 0.57 P = 0.0058	r = 0.64 P = 0.0012	r = 0.56 P = 0.0070	r = -0.09 P = 0.6969
OTU16 (*Eubacterium limosum* 93%)	r = -0.35 P = 0.0118	r = 0.48 P = 0.0241	r = -0.27 P = 0.2166	r = -0.30 P = 0.1759	r = -0.34 P = 0.1254	r = -0.29 P = 0.1876	r = -0.01 P = 0.9743

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Chapter 6

Discussion

Diet has long been considered a major factor affecting host metabolism and a driver of metabolic aberrations. The GM been recognized as a contributing factor to host metabolism and metabolic aberrations. However, a large gap in knowledge exists in terms of 1) which bacterial populations can be affected by dietary components in humans, as most of our knowledge thus far stems from studies that used analytical methods that target specific microorganisms, and mainly the effect of prebiotics was investigated, 2) which bacterial populations can impact host metabolism, and 3) the directionality of the associations between GM and health/disease. The studies presented in this thesis address these issues. Human trials were conducted to assess the impact of common dietary components on GM composition, resistant starches and whole grains. Furthermore, links between the GM and metabolic markers in humans were investigated. A series of animal experiments also helped us broaden our understanding of the impact of diet on the GM and host metabolism in the context of dyslipidemia, and allowed us to assess directionality between improvements in host lipid metabolism and the GM.

6.1 Effect of diet on gut bacterial populations

6.1.1 The GM composition in humans can be extensively altered by diet

The human trials conducted revealed that the GM composition can be substantially altered by dietary non-digestible carbohydrates. Resistant starches and whole grains induced GM modulations that encompassed bacterial populations from the phylum to the species level. Numerically, the most significant compositional shifts were prompted by the consumption of RS4, which in some subjects increased ten-fold the

abundance of *Bifidobacterium adolescentis*, making it the most dominant bacterial species during this dietary treatment for these subjects. Overall, whole grain intake excerted milder modulations of gut bacterial populations than RS. The populations most consistently affected by whole grains across the subjects were *Eubacterium rectale* and *Blautia wexlerae*.

The substrates investigated in these studies had differential outcomes in terms of community diversity within the host. Dietary incorporation of whole grains singificantly increased the α-diversity of the gut ecosystem, while neither RS2 nor RS4 affected α-diversity of the human GM. The distinct impact of the substrates on community diversity is likely to be reflective of the food webs that these substrates generate in the GIT. Fewer bacterial groups are likely to benefit from RS intake compared to whole grains. First, whole grains contain a more diverse profile of non-digestible compounds, which can potentially be degraded by a larger number of bacteria and consequently increase diversity in the ecosystem. An alternative or complementary possibility is that RS is more efficiently uptaken by its primary degraders, resulting in a smaller amount of substrate available for cross-feeding, which would restrain bacterial diversity in the GIT.

Although intake of dietary carbohydrates stimulates growth of members of the Firmicutes and reductions in Bacteroidetes (Duncan et al., 2008), the trials revealed that these generalizations do not apply to all NDC. Intake of RS4 significantly increased the abudance of Actinobacteria (through an increase in *Bifidobacterium adolescentis*) and Bacteriodetes (mainly due to the expansion of *Parabacteroides distasonis*), while significant reductions in the proportions of Firmicutes were observed. In the case of the whole grain intervention, the increase in Firmicutes and decrease in Bacteroidetes held true. However, in some subjects, large increases (over ten-fold) in *Bacteroides coprocola* were detected.

6.1.2 Why do we recurrently observe specific bacterial groups being enriched by intake of non-digestible carbohydrates?

Out of the 500-1,000 bacterial species associated with the human gastrointestinal tract, certain species are recurrently determined to be affected by NDC. Such is the case of bifidobacteria, Ruminococcaceae, and members of the *Clostridium coccoides-Eubacterium rectale* group. Until recently, this could have been attributed to the fact that taxa-specific analytical methods were used to investigate the impact of diet on the GM (i.e.: culture, FISH, qRT-PCR). But even community-wide approaches have determined proportions of these bacterial groups to be especially affected by NDC intake (Abell et al., 2008, Walker et al., 2011, Davis et al., 2011). For example, the bacteria mostly affected by RS and whole grains were *Bifidobacterium adolescentis* and *Eubacterium rectale*, respectively. Several explanations can account for this. First, given the individuality of the human GM in terms of the member species, changes in the abundance of 'core' members of the human gut microbiome are more likely be detected when the overall effects of diet are analyzed in a population of subjects. Both *Bifidobacterium adolescentis* and *Eubacterium rectale* have been identified as core species of the human gut microbiome. Although being a core species is an important factor for an organism to be idientified as a 'responder' organism across subjects, 60-90 gut bacterial species have been determined to be part of the human core (Qin et al., 2010, Willing et al., 2010), which means other factors need to be considered. A second determining factor could be the trophic level at which the organism utilizes the substrate could potentially determine its chances of having a significant increase in abundance. Only a subset of the species in the GIT are primary degraders of NDC (Stecher and Hardt, 2008, Ze et al., 2012), thus, if intake of

the adequate substrate increases, it is easy to envision that this bacterial population would expand. The impact of organisms themselves on the environment can lead to new niches to develop. NDC utilization by primary degraders generates the release of smaller carbohydrate units including mono- and disaccharides, which can be utilized by secondary degraders. Because a larger number of bacteria can utilize these smaller sized carbohydrates, the effect of the substrate becomes 'diluted out' at lower trophic levels, and the abundance of these bacteria cannot be increased as easily. The same concept is valid from the perspective of specialists and generalists. Ecological specialists have traded-off their competitive abilities in other niches to have increased fitness in a particular niche (Kassen and Rainew, 2004). Thus, if the appropriate conditions are met, specialists will thrive in the environment. Thus, if the right substrate is provided for a niche specialist, its population will most likely expand. On the contrary, the fitness advantage of generalists resides on rapidly adapting to changing environmental conditions, making their populations resilient to temporal heterogeneity which allows them to maintain more stable populations (Kassen and Rainew, 2004). The presence of a particular substrate is not likely to induce important changes in the population density of generalists.

6.2 Impact of diet on population dynamics

Diet induced changes in the gut microbiome were prompt, reversible and varied across subjects.

6.2.1 Dietary induced changes in the gut microbiota composition occur fast and are reversible

The gut microbiota is characterized by a remarkable temporal stability (Franks et al., 1998, Zoetendal et al., 1998). Nevertheless, the work described in this thesis and that of other researchers has shown that GM composition in humans can be altered by specific dietary strategies (Abell et al., 2008, Davis et al., 2011, Walker et al., 2011). Based on our RS study, we knew that bacterial modulations can take place within a week. In order to investigate the necessary time for dietary substrates to induce changes in GM structure, two of the subjects that showed increases in *Bifidobacterium adolescentis* in response to the RS4 treatment were followed up on a second trial in which the same dose of RS4 (30 g) was consumed by the subjects for 10 days but fecal sampling was performed every other instead of weekly. The results revealed that shifts in the abundance of *Bifidobacterium adolescentis* occurred within 48 h of the initiation of the treatment, indicating that bacteria can readily adapt to the availability of the substrate in the environment (Figure 6.1).

6.2.2 Individual response to diet

Both human trials conducted revealed that modulation of the GM through diet varies across subjects. Given that each human harbors a different collection of microbes, this observation does not seem surprising, as in some cases the bacterium is simply not present/detected in the subject. However, even when a species is detected, the impact of a dietary substrate on the microbial population can be different. Several factors could hamper the bacterial growth on a substrate: 1) the strain(s) present in the individual do not have the enzymatic capacity to degrade the substrate, 2) environmental conditions are not favorable for the utilization of the substrate by the

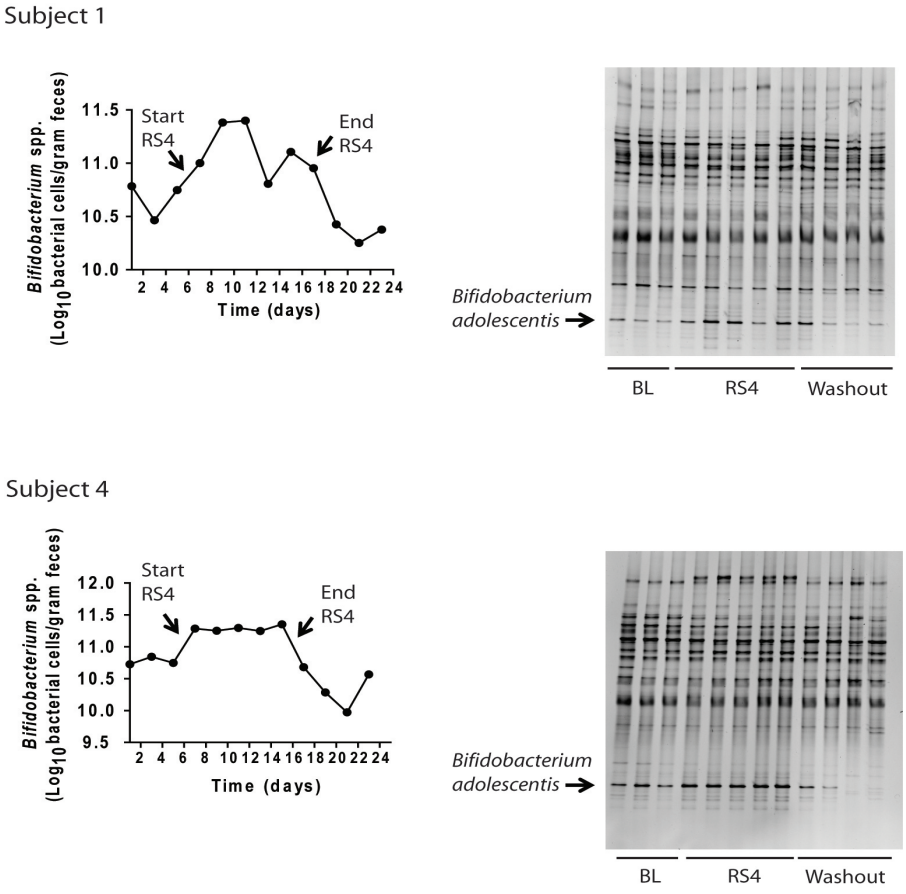

Figure 6.1: **Time-response in the increase in *Bifidobacterium adolescentis* due to RS4 intake**. Fecal samples from two subjects were collected every other day during a 6-day baseline period, 10-day RS4 treatment period, and 8-day washout period. *Bifidobacterium* spp. were quantified by qRT-PCR and the microbial community was profiled by DGGE.

bacterium (i.e.: pH, limitation of other nutrients necessary for growth, etc.), or 3) the bacterium relies on other bacteria for primary processing of the substrate and these bacteria are not present or subjected to factors 1) or 2).

In vitro studies have indicated *Bifidobacterium adolescentis* is a primary degrader of starch (McWilliam Leitch et al., 2007, Duncan et al., 2004). Therefore, it is likely that the failure to expand this bacterial population in some subjects was due to the enzymatic inability of the strain to utilize the substrate, or that the bacterium's utilization of the substrate was limited by environmental factors (factors 1 and 2). In the whole grain study, the hydrogenotrophic bacterium *Blautia wexlerae* was detected in all of the subjects but enriched only in a subset of the population when WGB was consumed. This species does not have the enzymatic capability to degrade β-glucans itself but can use hexoses, H_2 and CO_2 to grow (Figure 1.1). Because *Blautia* spp. is at lower trophic levels in the utilization of carbohydrates, unsuccessful enrichment of this species is most likely to occur due to unfavorable environmental conditions, or because microbial partners at higher trophic levels were unable to utilize the substrate.

Moreover, with approximately 160 bacterial species in an individual, there is high competition for substrate utilization. The lack of success to stimulate a particular bacterial species might also reside on other members of the GM being more competitive in substrate utilization, and depleting the substrate for the targeted GM member.

6.3 Diet, health and the gut microbiome

As previously discussed, the gut microbiome is host specific, temporally stable, and susceptible to host and environmental factors. At the same time, the gut microbiome

and the environment affect host physiology, metabolism and the immune system (Figure 6.2). The GM has been identified as a causative factor of metabolic aberrancies in the host (Cani et al., 2007, Bäckhed, 2011), which has prompted research to explore how the GM can be modulated to improve host heath. One of the main goals of our studies was to investigate how diet influenced changes in the GM composition and host metabolism, and how these changes were interrelated.

In three of the studies presented in this thesis we had the opportunity to evaluate the associations of bacterial taxa and host metabolism. The data revealed that dietary carbohydrates and lipids can significantly impact host metabolism. Remarkable correlations were observed between members of the families Coriobacteriaceae and Erysipelotrichaceae with the host lipidome. Importantly, these associations have also been determined by other researchers (Ravussin et al., 2012, Claus et al., 2011). Moreover, we were able to determine that whole grain intake induced glycemic improvements and anti-inflammatory effects on the subject population, and that some of these events were linked to changes in bacterial populations. From this study, we could also conclude that the abundance of the family Ruminococcaceae is negatively associated with several host inflammatory markers (IL-6,hs-CRP and LBP).

6.3.1 Directionality of the host-microbes interactions in response to diet

The dietary lipid content has been shown to affect the GM in both humans and animals (Hildebrandt et al., 2009, Ravussin et al., 2012, Murphy et al., 2010, Ding et al., 2010, Fava et al., 2012, Ley et al., 2006, Wu et al., 2011). However, because dietary lipids induce changes in the hosts' lipid metabolism, difficulties exist in differentiating the effects of diet from the potential impact of host metabolism on the GM commu-

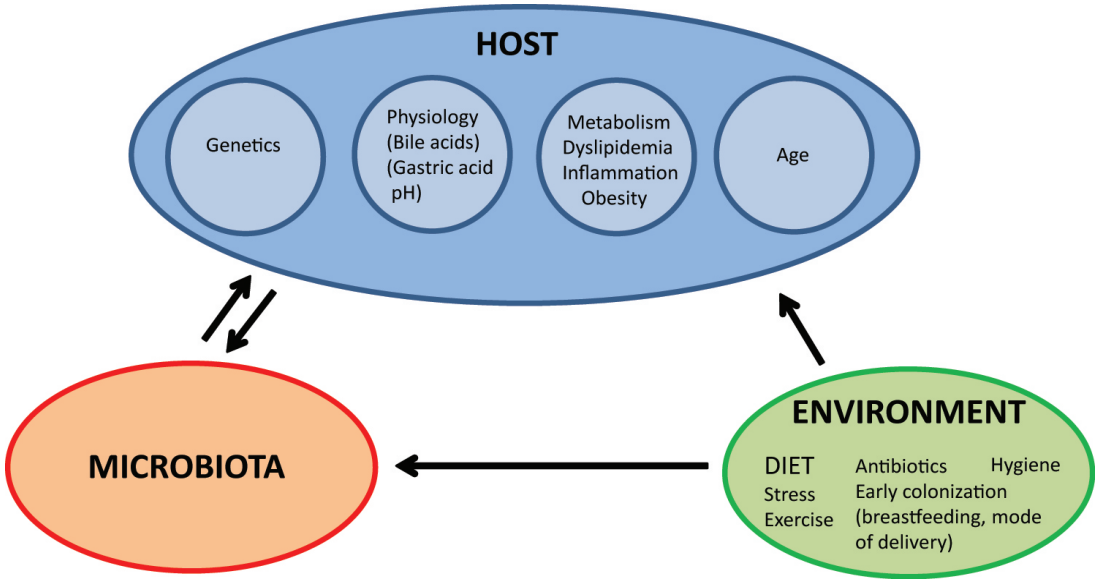

Figure 6.2: **Interactions between the host, microbiota and environment**. Diagram showing important environmental and factors affecting the gut microbiota composition

nity. Recent research has concluded that it is mainly metabolic changes in the host induced through a high fat diet that drive the observed bacterial dysbiosis (Ravussin et al., 2012, Hildebrandt et al., 2009), however, other studies have determined that it is the energy intake that shapes the GM (Murphy et al., 2010). Lack of clarity on this issue arises due to the gap in knowledge of the mechanisms underlying the dietary fat-microbiome-host metabolism interactions. One of the main findings in this thesis was the identification of cholesterol concentrations in the GIT as a potential modulator of the GM through an inhibitory effect on bacterial populations.

In the human trial conducted, although directionality of the associations between shifts in the microbiome and metabolic and inflammatory changes could not be determined, the GM alterations are likely to result from the increased NDC intake. The bacterial taxa affected were determined to either have the enzymatic capability

to degrade the substrates (i.e.: genes encoding for β-glucanase activity), or to feed off by-products of bacterial fermentation of NDC.

Overall, our data suggests that while dietary non-digestible carbohydrates directly modulate the gut microbial community dietary lipids shape the GM composition mainly through their impact on host metabolism.

6.3.2 Manifestation of our metabolic and inflammatory health status on the gut microbiome

The data obtained in these studies suggest that the abundance of certain bacterial populations could be linked to the health status of the host. For instance, in both hamsters experiments members of the Coriobacteriaceae and Erysipelotrichaceae families were associated with a dyslipidemic phenotype. Moreover, these bacterial taxa have been recurrently identified to be altered in dyslipidemic animals or animals in a high-fat diet (likely to have an aberrant lipid metabolism) (Claus et al., 2011, Ravussin et al., 2012). In humans, an inverse link between the abundance of Ruminococcaceae and host inflammation was determined (measured by IL-6, LBP and hs-CRP). Could these microbial patterns be a reflection of host health aberrancies? Which begs another question: could altered bacterial populations serve as diagnostic tools of metabolic disorders? Given that well characterized metabolic markers already serve as good indicators of metabolic disease, altered bacterial patterns could be of use if they could be observed prior to clinical manifestation of disease, a topic that could be further investigated.

6.4 Perspectives

6.4.1 Is our gut microbiome in permanent dysbiosis?

Dietary habits in Westernized societies greatly differ from those that we have evolved to (Leach and Sobolik, 2010). Humans have transitioned from a diet high in legumes, fruits, vegetables, nuts, and grains to consumption of highly refined processed foods with little dietary fiber content. It is estimated that our hunter-gatherer ancestors had a daily dietary fiber (including RS) intake of approximately 150-225 g, while today's estimated dietary fiber intake ranges between 13-18 g/day (Dietary Reference Intakes - USDA). Given the large effect of NDC in our gut microbiome, the marked reduction in fermentable substrates is bound to affect our GM composition and community assembly. Is it possible that individuals living in Westernized societies are in chronic dysbiosis? Differences in the GM between individuals from Westernized and non-Westernized societies suggest this is the case (De Filippo et al., 2010, Wu et al., 2011). Moreover, the two human trials conducted as part of this thesis used dietary components that were greatly consumed by our ancestors, whole grains and resistant starch (Leach and Sobolik, 2010). Their consumption led to pronounced alterations in the GM of individuals, especially of *Bifidobacteria* and *Eubacterium rectale*. Bifidobacteria have been shown to possess anti-inflammatory capacity (Wang et al., 2011, Gad et al., 2011, Fanning et al., 2012, Lyons et al., 2010), while *Eubacterium rectale* produce butyrate in the GIT, also considered anti-inflammatory (Segain et al., 2000, Russo et al., 2012). These observations lead us to speculate whether diet-host-microbe interactions emerged during evolution to control inflammation.

6.4.2 Predicting shifts in microbial composition in response to dietary interventions

It is clear that the GM is a complex ecosystem, with a large number of members that interact with each other, the host, and are further subjected to environmental perturbations. In this elaborate scenario, is it realistic to predict the modulations taking place in response to a dietary treatment?

Although researchers have been able to predict diet-induced shifts in the microbial community structure based on the enzymatic carbohydrate utilization capacity of organisms in simplified gnotobiotic animal models with controlled substrate supplies (Sonnenburg et al., 2010), human trials such as ours have proved that the reality of the gut ecosystem is too complex to allow accurate predictions to be made. Based on our data, generalizations as to whether a certain substrate can impact a specific gut bacterium cannot be made based solely on its enzymatic capability to degrade the substrate. A clear example is the case of *Bacteroides thetaiotaomicron*, which has the most ample repertoire of carbohydrate utilizing enzymes yet described in a gut organism, many of which are involved in the adherence, hydrolization and translocation of starch (Xu et al., 2003). Despite the prevalence of this organisms in humans, *in vivo* studies have not reported increases in the abundance of this microbe in dietary interventions with resistant starch (Abell et al., 2008, Walker et al., 2011). Similar observations were drawn from *in vivo* human trials that analyzed the impact of GOS on the GM (Davis et al., 2011) and in our own RS study here presented, despite the known α-amylase activity of numerous gut bacteria (Salyers et al., 1977). These observations reveal the need for human trials to genuinely understand the impact of dietary strategies on the GM.

Moreover, the individuality of the GM in terms of composition and response to dietary

interventions evidenced in these studies, indicate that generalizations as to the effect of foods and food components cannot be accurately made across individuals. If a specific bacterial population would be intended to be enriched in an individual, more personalized approaches would prove more successful. For example, *in vitro* fecal fermentations testing a panel of substrates could be performed to determine which substrates can elicit the growth of a specific bacterial group, using fecal material from that particular subject. Even this approach is not infallible, as *in vitro* conditions cannot completely replicate the *in vivo*. Another strategy could consist of having the individual undergo a series of dietary interventions with NDC and analyzing the GM community to determine which substrates could have the desired impact.

6.5 Concluding remarks

The studies presented in this dissertation provided insight into the diet-microbe-host interactions.

The use of community-wide deep sequencing techniques to characterize the GM enhanced our understanding of the effects of diet on the gut ecosystem in many regards: 1) the impact of diet on dozens of bacterial taxa and ecosytem diversity could be investigated, 2) knowledge was obtained on the interactions between bacterial members, and among bacterial populations and host metabolism, and 3) individuality of the response to diet and temporal dynamics could be studied.

Two human trials were conducted to evaluate the impact of different types of resistant starches (types 2 and 4) and of whole grains (brown rice and barley) on the GM. These studies revealed individuality in the response of the subjects to the dietary treatments. Although several bacterial populations stimulated by these dietary components were coherent across subjects, a high degree of variability was observed

among individuals in terms of the magnitude of the changes introduced and the bacterial groups affected. The data from the human trials also enabled us to conclude that changes in the gut microbiome composition were reversible and developed promptly after treatment commencement.

Animal experiments in a hamster model of hypercholesterolemia provided the opportunity to evaluate the effects of dietary lipids on the GM and the host lipidome. Inclusion of grain sorghum lipid extracts and plant sterol esters greatly modulated the GM community. Furthermore, remarkable correlations between GM compositional shifts and host lipidome were revealed. Mathematical modeling of these interactions suggested that some bacterial populations were inhibited by the cholesterol concentration in the GIT. Diet induced alterations in the cholesterol metabolism can therefore shape GM composition.

In summary, these studies allowed us to conclude that while dietary non-digestible carbohydrates are able to directly modulate the GM composition, it is likely that dietary lipids exert their effect on the GM community by affecting the host metabolism. In particular, we determined that cholesterol concentrations in the GIT might constitute a potential mechanism by which host metabolism shapes the GM.

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